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ON THE SURFACE TENSION CHANGES WHICH UNDERLIE MUSCULAR AND AMOEBOID MOVEMENT.

by

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(From the Department of Zoology, University of Melbourne).

(Submitted for publication 22nd January, 1926.)

The theory that alterations of surface tension underlie muscular and apparently protoplasmic movement in general has been developed by D'Arsonval, by Quincke, and notably by Julius Bernstein (3). The work of D'Arsonval is of especially great interest, in that it involved the application of the same principle as that which underlies the action of the capillary electrometer, i.e., a surface tension change accompanying an alteration in the quantity of electricity condensed as a double layer at the interface of two fluids.

The first attempt to fit a surface tension scheme into precise histological observations was made by T. B. Robertson (6). This theory, which is based on the description of a contracting sarcomere as given by Schäfer, depends on the same principle as that suggested by D'Arsonval, namely, a change of surface tension accompanying an alteration of charge on a condenser, as the excitation wave travels through the fibre; the condenser being constituted by the electrical double layer which must exist at the surface of the hyaloplasm, if this hyaloplasm contains ion-proteid compounds. I have proposed a mechanism, depending upon the same principle, but based on microscopic observations which led to conclusions somewhat different from those of Schäfer (8, 9). The conception involved in this theory is, that there occurs in muscle a change of surface tension accompanying an alteration of the quantity of electricty condensed at its surface, under the influence of lactic acid, liberated not directly on the surface, but in its neighbourhood. A theory, not differing essentially from this has also recently been suggested by A. V. Hill (5).[1] In the present paper I wish to discuss this surface tension scheme in greater detail.

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[1] Hill has shown that the quantity of lactic acid formed by a muscle on excitation is quite inadequate to cover, even in a layer one molecule thick, the free surface necessary to develop a tension of the order observed in a muscle twitch. But in denying that surface tension can therefore play any part in the process of contraction, the fact seems to have been overlooked that a surface tension change can be produced by means other than the spreading of a substance

The facts upon which the theory is based are the following:

1. The unit of contraction of the muscle fibre is the sarcomere, and not an invisible ultramicroscopic colloidal complex. This is proved by the fact that the sarcomere can be observed to undergo contraction as a result of a change in position of part of its fluid contents.

2. The middle third of a sarcomere can be shown to contain a substance which does not undergo change in position as the sarcomere passes from the relaxed into the contracted condition. This substance is recognizable by the fact that after fixation it is still doubly refracting to polarized light.

3. The sarcomere also contains a fluid (hyaloplasm), recognizable because of its affinity for haematoxylin. In the *relaxed* muscle this fluid occupies the region of the sarcomere (middle third), which is doubly refracting; it is also found in very small quantity at the ends of the sarcomere, immediately adjacent to Krause's membrane (see figure 3). For convenience the hyaloplasm of the middle of the sarcomere and at the ends will be referred to respectively as the movable and the residual hyaloplasm. Under very high magnification delicate streaks of a similarly stainable material are seen, connecting the movable with the residual hyaloplasm at either end of the sarcomere. These streaks are especially well seen in flying muscles of insects, where Schäfer first observed them. I have also seen them in leg muscles of insects (see figure 3).

When now the sarcomere is examined in the contracted state the movable hyaloplasm is seen to have retreated entirely from the middle of the sarcomere (which still remains doubly refracting, however), and to have accumulated at the ends of the sarcomere, in the region previously occupied by the residual

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over the active surface; for example, in the capillary electrometer, the surface electrification which produces the surface tension change is brought about by the application of a potential difference, at points distant from the active surface; and it seems to be precisely on this principle that the surface tension machine of muscle acts.

A theory of muscular contraction has recently been proposed by W. E. Garner (Proc. Roy. Soc. (B), 99 (1925), p. 40), in which it is maintained that muscular contraction is the result of a chemical interaction between the lactic acid and the substance of the anisotropic segments of the myofibrils, the liquid crystal structure of the latter being converted at its surface into a solid film, with the production of a tension parallel to the axis of the fibril. However successful this theory may be in explaining the process of shortening of a muscle, it encounters the fatal objection that histological observation definitely contradicts it. If numerous microscopic preparations of muscle fibres (especially insect muscle fibres) are made, sooner or later one will encounter the good fortune to obtain a fibre in which a contractile wave has been fixed. If the sarcomeres of successive striations along such a contractile wave be examined, the whole process of contraction can be followed without difficulty, and in such preparations it can be observed with unmistakable clearness that the process of contraction is the result of the movement of a fluid *within* the sarcomere. The theory as developed by Garner leads to the conclusion that an individual sarcomere homogeneous to microscopic appearance, merely undergoes a shortening, while retaining its homogeneous appearance; microscopic observation shows beyond doubt that such is not the case.

A second difficulty that Garner's theory will encounter is that the sarcomeres should tend to swell in their mid-region (anistropic portion) when they contract, i.e., a cylindrical sarcomere will become cask-shaped. Microscopically it is found that precisely the opposite occurs (see, for example, figures 1 and 2).

hyaloplasm alone (compare figures 1 and 2; also 3 and 4). When successive sarcomeres in a fixed contractile wave are examined, all intermediate stages between complete relaxation and contraction can be observed.

It might be urged that the change in position of the movable hyaloplasm is only an apparent one, and that an alteration in the acidity of the middle and ends of the sarcomere underlies the reversal of the staining reaction, but this is disproved by the fact that the tissue, during its preparation for microscopic examination, undergoes treatment with acid reagents, etc., so that any such variation of acidity of its parts will be abolished long before the tissue is stained.

4. The following observation is of especially great importance: If successive sarcomeres along a fixed contractile wave are examined a very considerable movement of the hyaloplasm can be seen to have occurred, without the sarcomere having undergone any visible contraction. From this we must conclude that the movement of the hyaloplasm is not caused by contraction of the sarcomere, but that contraction of the sarcomere occurs only after a sufficiently extensive movement of hyaloplasm has occurred.

5. Bernstein (3) gives reasons for concluding that the surface tension change cannot occur on the surface of the myofibrillae, but that a larger, free surface, situated within the fibrillae, is necessary. Where is this free surface situated? Is it in the doubly refracting middle of the sarcomere, or is it to be sought for at the ends, adjacent to Krause's membrane? The histological observations are decidedly in favour of the view that it occurs in the middle of the sarcomere. Firstly, there is a substance present in this situation in large enough quantity to be recognizable by its double refraction in polarized light; and this doubly refracting substance cannot be the hyaloplasm, since its optical properties remain unchanged, even after the hyaloplasm has retreated from it. Secondly, the volume of the hyaloplasm in the contractile sarcomere appears to be considerably smaller than in the relaxed sarcomere (compare figures 3 and 4); in the contracted sarcomere it is also considerably more strongly staining than in the relaxed sarcomere. These observations find a ready interpretation if we assume that the doubly refracting material, which is relatively weakly staining, is porous, and presents a large free surface, into which the hyaloplasm extends.

We therefore regard the middle of the sarcomere as consisting of a highly porous substance; its cavity is connected by means of very fine capillary channels (observable under the microscope as the blue-staining longitudinal streaks above referred to), with a space at either end of the sarcomere, which presents relatively little free surface, and is probably to some extent collapsible; it might be in the form of a series of collapsible sacs, or of a loose spongework. During contraction the hyaloplasm is drawn from the doubly refracting porous mid-region of the sarcomere, through the fine longitudinal channels, into the collapsible spongework at the ends of the sarcomere. This spongework is dilated, and the sarcomere thickens at its ends, and therefore shortens. Relaxation occurs when the hyaloplasm moves back into the central porous substance.

If we could locate the large free surface at the ends of the sarcomere, rather than in its middle, it seems that a simpler surface tension scheme than that outlined below could be conceived, for in this case we should have two large porous chambers, communicating by very fine longitudinal capillary tubes with a central reservoir. If the hyaloplasm were in contact with the entire free surface of the porous substance, then lowering the surface tension between the two would tend to cause an increase of their area of contact, i.e., swelling of the base of the sarcomere would occur, hyaloplasm would be drawn in from the central reservoir, through the lowering of pressure as dilatation occurred, and the chief histological features of contraction could be accounted for. But all the histological evidence points to the conclusion that the large porous surface is situated in the middle of the sarcomere, and not at its ends, and this hypothesis need no longer be entertained. This view would further imply that the movement of the hyaloplasm was a secondary consequence of contraction; we have seen above that the opposite is the case.

6. During excitation lactic acid is liberated; we cannot, however, say, *a priori*, whether this acid will cause a decrease or an increase of the surface tensions existing within the fibre, since it may cause a decrease by charging a surface, or an increase by depolarizing a previously charged membrane. I have assumed that the lactic acid is liberated in the interfibrillar spaces, and that it diffuses on to the walls of the sarcomere, exerting its effects, not by coming into direct contact with the active surface, but rather by polarizing the surface through its electrostatic action (9, 10). This theory is in harmony with the conclusion recently reached by A. V. Hill, namely, that lactic acid acts, not by spreading over the active surface, but by altering the degree of polarization of a membrane, and thereby changing the strain on this surface in a manner analagous to the removal of a strain from a condenser on discharging it (5).

Before considering the surface tension mechanism which seems to underlie muscular contraction, a physical analogy may first be discussed. Suppose one end

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REFERENCE TO PLATE.

Fig. 1. Portion of uncontracted body muscle of full-grown larva of a beetle (*Cnemoplites blackburni*) in thin longitudinal section (X 2000). The individual sarcomeres are clearly seen. Z indicates Krause's membrane.

Fig. 2. The same, in a contracted state. Note the "reversal" of the striations. (X 2000.)

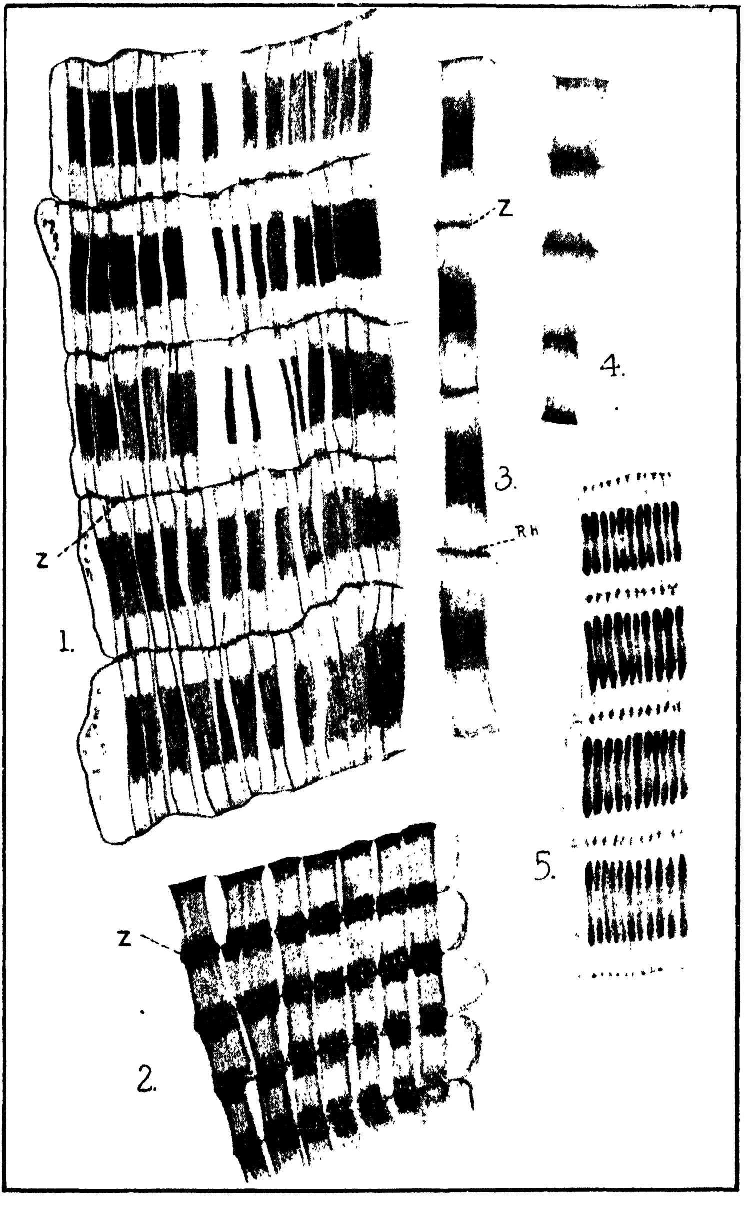
Fig. 3. Portion of a relaxed myofibril from same (X 3400). Z, Krause's membrane; R.H., residual hyaloplasm.

Note that the residual hyaloplasm is connected by very fine streaks of stainable substance with the movable hyaloplasm, which comprises the striation.

Fig. 4. The same, in a contracted state (X 3400). The narrowing of the fibril towards its lower end is due to its being cut tangentially.

Fig. 5. Part of leg muscle of an adult chalcid wasp (*Nasonia*).

Note Hensen's line; also the residual hyaloplasm. (X 2000.)



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of a capillary tube is dipped into a dish of water, and the conditions so arranged that gravity will not prevent water from moving along the tube (figure 6). Consider first the case in which the liquid (water) wets the solid (glass) entirely. When the capillary tube is dipped into the water a film of water spreads over the surface of the glass; the surface tension between the air and the water in the tube tends to reduce this surface to a minimum, and the water is drawn along the tube. Since gravity is not acting upon the water column, the water will eventually be drawn right along the tube. Suppose now that previous to dipping the tube into water a distensible rubber cap (A) had been fitted to its free end; if the tube were now dipped into the water, the water would be drawn into it, until the surface tension force between the air and the water within the tube was equal to the gradually increasing elastic resistance of the rubber cap. Since the total superficial energy of a fluid is proportional to its surface, the degree of distension of the rubber cap could be greatly increased by making the area of contact between the air and the water in the tube very large, such as by having this region in the form of a spongework, rather than of a simple tube. If the surface tension between the air and the water were now decreased, then the elastic recoil of the rubber cap would overcome the surface tension force, and the water would be forced backwards, until the elastic resistance of the rubber cap was equal to the opposing surface tension force. When the tension was again increased water would again move towards the cap, which would distend. Suppose further that the reservoir of water in the model were replaced by an elastic sac of such a kind that it was distensible only transversely. In this case, if we lower the surface tension between the air and the water, the rubber sac would markedly dilate, while the cap A would tend to collapse; furthermore, the dilatation of the sac would be mainly at right angles to the direction of maximal collapse of the cap.

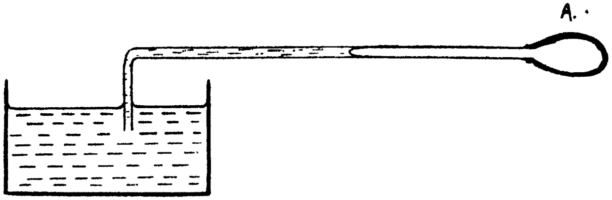

Fig. 6

If we had chosen a liquid that did not completely wet the glass, the results would have been the same, except that the area over which the surface tension force could act would be very considerably smaller.

The above model seems to illustrate well the principle on which the surface tension machine of the muscle acts. The rubber sac represents the spongy tissue at the two ends of the sarcomere, which contains the residual hyaloplasm. The capillary tube of the model is represented in the sarcomere by the very fine capillary tubes that connect the cavity of the doubly refracting porous material with the loose, spongy tissue at the ends of the sarcomere, and also by the porous material itself. The analogy is incomplete to the extent that the rubber cap in the model becomes in the sarcomere the cavity of the porous material, which at the same time exhibits the large surface area. The hyaloplasm is represented by the water. The air in the model, I previously supposed, was represented by water vapour. I do not believe that this suggestion is a good one, for one does not expect to find free vapours within living tissues. I would here suggest rather that the third surface, which is necessary for the working of the surface tension machine as it seems to occur in muscle is presented by a liquid. It may seem that this is an unjustifiable assumption; nevertheless, there is some direct microscopical evidence in its favour. It is often possible to observe, in the middle of the doubly refracting region of the sarcomere, a light weakly staining area (Hensen's line) (see figure 5). In the flying muscles of insects it is indeed extremely prominent. I would suggest that this light staining area in the middle of the porous material is occupied by the third substance, necessary for the working of the surface tension machine. It is clear, at the same time, that if the hyaloplasm entirely wets the walls of the sarcomere, a well-defined pale region in the middle of the dark portion in the relaxed sarcomere might not necessarily be distinctly visible.

The working of the surface tension machine would then be as follows: The contracted sarcomere undergoes relaxation as a result of an increase in the surface tension between the hyaloplasm and the fluid which occupies the region of Hensen's line. The hyaloplasm is drawn further into the doubly refracting porous substance, and since a very considerable free area separates the two fluids in this region, a considerable force, causing a distension of the middle of the sarcomere, will be developed. This will be counteracted by the elastic rebound of the porous material, and a position of equilibrium will be attained. When the relaxed sarcomere is to contract, a fall in the surface tension between the two liquids occurs; the elastic recoil now overcomes the opposing surface tension force, and the fluid is driven out of the porous material, through the fine capillary tubes, into the collapsible sacs or spongework at either end of the sarcomere. If these sacs are of suitable structure, they will thicken from side to side; this will cause the ends of the sarcomere to distend, so that the sarcomere as a whole will shorten.

Concerning the manner in which the lactic acid brings about the surface tension change no definite facts are available, but the following hypothesis may be entertained. It seems improbable that the delicate contractile machine should receive and store the actual fatigue products during its activity; we shall assume that the main chemical reactions that bring about contraction occur in the interfibrillar space.

Now suppose that the wall of the sarcomere constitutes a membrane, semipermeable in such a way that it will permit diffusion of the small hydrogen and hydroxyl ions, etc., but not of the large ions, such as protein, and perhaps even lactate ion. Let us suppose the hyaloplasm to be of such a nature that if it could be completely dissociated, it would have a preponderance of negative hydroxyl ions. Since, however, the hydroxyl ion is but weakly dissociated in protein, therefore the interface between the hyaloplasm and the walls of the sarcomere will be but feebly polarized. When free lactic acid is liberated on to the sarcomere the hydrogen ions, diffusing into the thin walls of the sarcomere, will combine with the small quantity of free hydroxyl ion present there. More hydroxyl ion

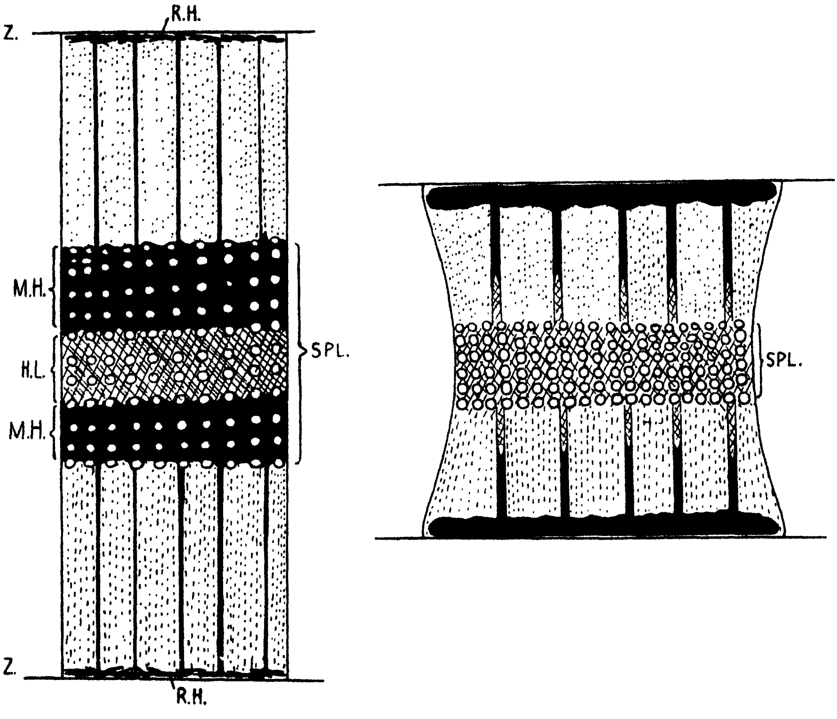

Fig. 7. Diagram of two sarcomeres, in the relaxed and contracted conditions respectively. Z, Krause's membrane; SPL., doubly refracting porous material ("spongioplasm"); H.L., fluid comprising Hensen's line; M.H., movable hyaloplasm; R.H., residual hyaloplasm.

The diagram attempts to show the collapsible spongework adjacent to Krause's membrane; also the stretching of the elastic porous material (SPL.) of the relaxed sarcomere. On the other hand, it fails to show how the surface of contact between the two fluids H.L. and M.L. increases when contraction occurs. Since the fluid M.H. is regarded as completely wetting the porous material, it will extend as a thin film into the porous material, even when the sarcomere is contracted; but it is not possible to represent this in the diagram.

will be formed and combine with the hydrogen ion. The sarcomere thus presents a condition analogous to a charged condenser; the hyaloplasm will be charged, and this charge will be held by the lactate ions on the outer walls of the sarcomere. If we must conclude that the walls of the sarcomere are too thick to permit the hydrogen ions to reach the hydroxyl ions, then we should have to regard the sarcomere wall as permeable to the lactate ion, and place the semipermeable membrane at the molecular interface between the hyaloplasm and the sarcomere wall.

The charging of the hyaloplasm will result in the lowering of its surface tension, and contraction of the sarcomere will take place, through the action of the surface tension mechanism above discussed.

Meanwhile basic creatine, formed on the second excitable membrane (11), will diffuse on to the walls of the sarcomere, but since its rate of diffusion is much slower than that of the lactic acid (11), it will reach the sarcomere later than will the acid. The hydroxyl ions will enter the walls of the sarcomere; in the interfibrillar space creatine lactate will form, and the condenser will be discharged. The surface tension of the hyaloplasm will again rise, and the sarcomere will once more relax.

The interpretation which I previously placed on the shape of the twitch curve (9) would have to be modified to some extent. The hydrogen ions, suddenly penetrating the membrane of the sarcomere, will explain the sudden rise of the twitch curve; the slower fall would be due to the slower rate of diffusion of the creatine; and the gradual sagging away of the contraction towards its end will be the result of the falling off of the rate of diffusion, as the concentration of the diffusing *base* decreases. The interpretation which I have placed upon the delay in the rate of relaxation that accompanies an isometric twitch carried out under a condition of initial elongation (Hartree and Hill) will in no way be affected; it is due merely to a proportional increase in the longitudinal distance through which the base must flow, as the distance between the two excitable membranes is increased. The theory also offers, as I have previously tried to show (10), an obvious explanation of the fact observed by Heidenhain, Fick, and recently by Fenn (4), that the amount of heat liberated by a muscle when lifting a weight is greater than that liberated when contracting isometrically, the excess heat liberated being an internal frictional effect, capable of being estimated in terms of Poiseuille's formula. Lest it might be urged that Poiseuille's law is valid only for thin capillary tubes, it may be pointed out that Bartell has recently found it to hold even for the case of fluids forced under pressure through a porous filter impregnated with copper ferrocyanide (2). That much of the heat liberated by a contracting muscle during the performance of work is of "thermodynamic" origin has also been suggested by Azuma (1).

On Amoeboid Movement.

Functionally the extrusion of a pseudopod by an amoeboid cell is analogous to the contraction of a sarcomere; in either case an external resistance is overcome. But the resemblance appears to be a much closer one.

The formation of pseudopodia in leucocytes has been described by Schäfer, and illustrated by some beautiful micro-photographs of the living cell (7). The middle region of the cell is occupied by a delicate lace-like reticulum, occupying the region usually described as endoplasm. When a pseudopod is about to be extruded, a granular fluid (ectoplasm) flows out from the reticulum, and the cell wall is caused to protrude in this region. The pseudopod grows in size as the ectoplasm flowing into it increases in quantity; finally the endoplasmic reticulum also enters the pseudopod, but this is clearly a secondary effect. The pseudopod is retracted again by the ectoplasm being withdrawn into the endoplasm.

The analogy to a contracting sarcomere is obviously a close one; the endoplasmic reticulum will correspond to the doubly refracting porous substance of the sarcomere; the granular ectoplasm flowing from the reticulum into the region between the cell wall and the endoplasm is obviously analogous to the movement of hyaloplasm in the sarcomere from the central porous material into the loose spongework at the ends of the sarcomere. The third surface necessary for the working of the surface tension machine would be provided, in an amoeboid cell, by the fluid which pervades the endoplasmic reticulum, after the ectoplasm has been extruded. The presence of this fluid (which would correspond to the fluid forming Hensen's line in the sarcomere) is easily recognizable through the fact that it contains numerous large granules usually undergoing marked Brownian movement.

Underlying the protrusion and retraction of a pseudopod, then, there would be the following processes: When a pseudopod is to be extruded there occurs a lowering of the surface tension between the granular ectoplasm and the fluid content of the endoplasm; and on account of the elastic recoil of the reticulum, the ectoplasm will gradually flow out from the reticulum and will cause a bulging of the cell wall, resulting in the formation of a pseudopod. When the surface tension is again increased, the ectoplasm is once more withdrawn into the reticulum; the surface tension force between the surrounding water, or medium, and the wall of the cell will tend to reduce the area of contact to a minimum, and, the opposing force having been removed, the pseudopod will be obliterated.

In the case of excised muscle this latter force is not acting; which explains the fact that a muscle undergoes only incomplete elongation when contraction ceases, unless an external force be applied to elongate it again.

SUMMARY AND CONCLUSIONS.

1. The unit of contraction of a muscle fibre is the sarcomere.

2. The contractile machine consists of two fluids in contact, and supported within an elastic porous substance, whose cavity communicates by very fine tubes with a collapsible spongework at either end of the sarcomere. When the surface tension between the two fluids is decreased, fluid is forced out of the porous substance, as a result of the elastic recoil of the latter, into the spongework, and the sarcomere thickens at its ends, and therefore shortens; when the tension is again increased, fluid is again drawn into the spongework.

3. The contractile machine works on the same principle as does the capillary electrometer, i.e., a change in the surface tension of a fluid, accompanying a change in its state of electrification. A mechanism is suggested whereby the lactic acid brings about this electrification; neutralization of the lactic acid by basic creatine abolishes the electrification, and the sarcomere again relaxes.

4. The same principle appears to underlie amoeboid movement in general.

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A NOTE ON THE ENZYMES OF THE HYDATID CYST.

by

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and Pathology, Melbourne).

(Submitted for publication 11th December, 1925.)

In a previous paper (3) a study of the micro-chemical reactions of the hydatid cyst wall was described. The next step in the investigation was to determine what enzymes are present in the cyst wall and in the fluid contained within its cavity. For this purpose it has been necessary to make use of two different methods of approach, one suitable for the study of enzymes in tissues, the other for enzymes in fluids. In connection with the former, the auxanographic or plate method, as described by Eijckman (5), working with bacterial enzymes, and by Buxton (1) in his studies on the enzymes of tumours, has been employed. In the latter case the ordinary laboratory methods could be used. The results obtained are only qualitative.

Methods.

(1) *For the Determination of Enzymes in the Cyst Wall.*

A series of freshly prepared agar plates were made in the manner described by Buxton (1), so as to test for diastase, lipase, protease, and oxidase. For detecting diastase, two media were used, one containing 0·5 per cent. starch, the other 0·25 per cent. glycogen. For lipase the media contained 1 per cent. butyrin and 1 per cent. olive oil. For proteases three media were prepared, containing 5 per cent. suspension of egg white, 10 per cent. sterilized milk, and 10 per cent. gelatin respectively.

In testing for oxidases the guaiac and peroxidase tests used by Buxton were employed, and plates containing 1 per cent. hydrokinone were found to be satisfactory, though the indo-phenol reaction could not be used on account of the rapid oxidation of the surface of the plates, which occurred without the addition of oxidase-containing substances.

Organs containing hydatid cysts were obtained from the abattoirs as soon as possible after the animals had been killed, so as to minimize post-mortem change and invasion of the cyst tissue by bacteria. The cysts were removed from these with strict aseptic precautions. All receptacles and instruments were sterilized in an autoclave, and great care was taken to prevent any contamination. That success was achieved is shown by the fact that in no case was there bacterial growth on the media employed. Many cysts were examined, chiefly from the liver, but also from the lungs, and fluid from both sources was tested separately.

The surface of the organ was first cauterized, and a deep incision made with a sterilized scalpel to expose the cyst, which was opened with a fresh scalpel after the fluid had been aspirated. The mother and daughter cysts were then removed with forceps, cut into very fine pieces with scissors, and ground up in a mortar with fine sand. Glycerin of 50 per cent. strength was added until a soft paste was obtained. Small pieces of this material were placed on the plates, which were incubated at 36°C. for three days and observed every twenty-four hours.

(2) *For the Determination of Enzymes in Hydatid Fluid.*

The methods employed were for the most part those described by Cole (4) in his text-book. In every case freshly prepared reagents were used. For diastase Sladden's modification (6) of Wohlgemuth's method was selected, and also the incubation of relatively large amounts of fluid (15 cc.) with 2 cc. of 0·1 per cent. solution of starch or glycogen, the mixture being titrated with N/10 sodium hydroxide before incubation to a pH of 6·7 using two drops of 1·0 per cent. neutral red as an indicator.

The presence of lipase was determined by incubation at 38°C., with olive oil, in the manner described by Cameron (2), and with butyrin. For proteases, Mett's method, with small tubes of egg white, and 0·4 per cent. hydrochloric acid, as well as incubation of varying quantities (up to 10 c.c.) of hydatid fluid with egg white, coagulated milk, and fibrin as substrates in the presence of 0·4 per cent. solution of sodium hydroxide were used.

Results.

The results of these various tests are presented in tabular form. In every case control experiments in which no hydatid fluid or tissue was used were negative.

	CYST TISSUE.			CYST FLUID.		
	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.
Diastase.						
i. Starch splitting	+	++	++	—	—	—
ii. Glycogen splitting	+	++	++	—	—	—
Lipase.						
i. Acting on Butyrin	—	S.	S.	—	V.S.	V.S.
ii. Acting on olive oil	—	S.	S.	—	V.S.	V.S.
Proteases.						
i. Egg albumen	—	—	—	—	—	—
ii. Milk	+	++	++	—	—	—
iii. Gelatin	—	V.S.	V.S.	—	—	—
Oxidases.						
i. Hydrokinone	—	V.S.	V.S.	—	—	—
ii. Guaiac test	V.S.	—	—	—	—	—
iii. Peroxidase test	V.S.	—	—	—	—	—

+ = reaction. — = no reaction. S = slight reaction. V.S. = very slight reaction.

It is therefore concluded that the chief enzymes found in the hydatid cyst wall are diastatic, that there are in addition a proteolytic enzyme which coagulates milk, a lipase (present only in traces), a protease which liquifies gelatin, and oxidases, all of these being much less in evidence than the former. In the case of the cyst fluid, except for lipase, which is present in exceedingly low concentration, enzymes are absent. The situation of the cyst makes no difference in this respect, although some evidence was obtained that the concentration of the enzymes varied according to the site of development of the parasite, the diastatic ferment being less easily demonstrated in cysts of the lung than in those of the liver.

The chief points of interest, apart from the purely observational nature of this work, are:

1. That the enzymes of the hydatid cyst are confined to the cyst wall.
2. That diastatic enzymes appear to be prominent, although, like most tissues, fat splitting, and at any rate, certain proteolytic ferments, are also present.
3. That hydatid fluid is, as regards enzymes, practically inert.

The first observation is interesting when one considers that the hydatid cyst is extremely common in the liver, one of the great storehouses of glycogen in the body. It is to be presumed that glycogen plays an important part in the nutrition of the parasite, and the variable amount of sugar found in the cyst fluid may result from glycogenolysis within the cyst wall.

The rich supply of glycogen present in the liver may account for the rapid development in that site of scolices into mature cysts. It is possible also that the presence of glycogen may play a part in the development of the parasites in other organs. Should it be possible to culture the parasite in vitro, whether in the form of a cyst or from scolices, an adequate supply of a suitable carbohydrate may prove to be an essential factor.

ACKNOWLEDGMENTS.

I have to gratefully acknowledge assistance from Dr. C. H. Kellaway (Director of the Walter and Eliza Hall Institute of Research), Dr. W. Davies (of the Organic Chemistry School), Mr. A. B. P. Amies (of the Dental Hospital), and Mr. Dickinson (of the Pathological Department at the University).

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STUDIES ON THE REFRIGERATION OF MEAT.

I. INVESTIGATIONS INTO THE REFRIGERATION OF BEEF.

by

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1. INTRODUCTION.

It is well known that beef which has been frozen slowly in air and then thawed loses from all cut surfaces a clear, reddish-coloured fluid. This exudation, known as "drip," contains about 9% protein, and its presence shows that the cycle of the changes of phase of the beef, from liquid to solid and back to liquid, produces definite alterations in the tissue structure, whereby water and protein are liberated. The work of the British Food Investigation Board (1), and of the German workers Plank and Kallert (11), has shown that the presence of drip is due to changes which have taken place during the freezing, and which are not reversed when the material is thawed. The same workers also showed that one of the factors affecting this change is the rate of freezing, and that much of the drip may be prevented by freezing at a sufficiently rapid rate. The British Board (2) have investigated the effects of different rates of freezing upon simple colloids, particularly protein sols and gels. They found that when a 12% gelatin gel was frozen slowly, for instance in air at —11°C., water separated from the colloid, froze as ice on the outside of the system, and was never completely reabsorbed on thawing. The residual colloidal mass was thus "dehydrated," and contained, in some cases, as much as 54% gelatin. When freezing was carried out more rapidly, ice crystals formed within the gel, and the quantity of water which separated when the mass was thawed was greatly lessened. When the gels were frozen in liquid air, the rate became so great that the whole froze as a "glass," and on thawing the material differed from the original gel only in being slightly more opaque. The general conclusion was that the less the amount of water separating out during the freezing, the greater was the degree of reversibility on thawing.

It was further found that a great deal of the water which had separated from the gels during slow freezing was reabsorbed by the gel subsequent to thawing, but this absorption was never complete. The freezing had apparently caused a loosening of the union between the dispersed phase and the water, and the resulting "dehydrated" mass was capable of imbibing water to a limited extent. The rate of this reabsorption, however, could be increased by raising the temperature, so that in the cases of hydrosols and hydrogels of protein it might be possible to obtain complete reversibility by a combination of rapid freezing and slow thawing, and a subsequent exposure to a temperature above 0°C.

More recent work, however, seems to show that there are other factors which operate in the change, one of which may be the actual temperature to which the colloid is cooled during the process. Thus Moran (8) states that when hens' eggs are frozen by exposure to temperatures above —6°C., complete reconstitution of both yolk and white takes place on thawing. If, however, the temperatures employed be below —6°C., or even if the eggs be frozen above —6°C., and later stored at —11°C., an irreversible change takes place, so that after thawing, the eggs have pasty yolks and turbid whites. In a review of the theories of the freezing of tissues, Plank (9) suggests that there may also be a similar critical temperature with muscle colloids. In a later paper (10), he points out that at the freezing point of muscle (approximately —1·5°C.) only 42% of the water is frozen, that a fall of temperature below this point causes a gradual separation of the unfrozen water from the colloidal mass, and that after thawing such water is reabsorbed only with difficulty. Consequently exposure to low temperatures would tend to produce an irreversible change.

The problem of refrigeration of meat is therefore a very complex one. Ox muscle may be regarded as consisting of a number of colloidal systems, each composed of the muscle plasma, bounded by a semi-permeable membrane (the sarcolemma); any theory of the freezing of tissues must take into account changes in both these elements during freezing and thawing.

The present paper records experiments carried out for the Committee for the Freezing of Beef* of the Australian National Research Council. The object of the investigation was to develop, if possible, such methods of treatment that the beef underwent no changes during the freezing process which could not be reversed during thawing. In addition, it was sought to obtain data on the rates of freezing and thawing which could be attained with pieces of material similar in size to those actually used by the trade (e.g., quarters of beef). This latter work was carried out at the suggestion of the British Food Investigation Board, which at that time had more or less confined its attention to the investigation of the freezing and thawing properties of small pieces.

The actual biochemical and histological work has been carried out by J. R. V. and the physical measurements by G. A. C.

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* It was at the suggestion of the chairman of this committee, Dr. J. A. Gilruth, that the investigation in Australia was commenced.

2. METHODS OF ATTACK.

(a) Methods of Freezing and Thawing.

In these investigations two methods were employed to vary the rate of freezing. In the first method, media at different temperatures and with different specific conductivities were employed, such as cold air and a calcium chloride ("brine") bath. Similar samples could thus be frozen at different rates, and when subsequently thawed under similar conditions, gave the same rates of thawing. By this method, however, the more rapidly frozen pieces were reduced to a lower temperature in the process than the others. As very low temperatures are stated to be one factor in changes occurring during freezing, this would tend to increase changes in the more rapidly frozen samples, and hence to lessen any differences between these and the more slowly frozen samples. This influence was avoided in the second method by employing the same freezing bath and varying the size of the samples. Pieces of the meat were cut exactly to fit rectangular tin containers of the same length and breadth, but varying in thickness. When immersed in the same bath these gave a series with gradually increasing rates of freezing. This method, however, introduced other differences, in that when the pieces were thawed under similar conditions, the time taken in thawing also varied with the thickness. In this case also, as will be seen later, slow thawing would tend to bring about reconstitution, and therefore the differences in thawing would again lessen any changes due to slower freezing. In both methods, therefore, the differences actually observed would be smaller than the changes due to the rate of freezing alone.

For the extreme rate of freezing, liquid air was employed. Different thawing rates were obtained by employing air or water as the medium.

(b) Measurement of Temperature.

It was essential in these experiments to have an accurate method of estimating the rates of freezing and thawing. In the earlier experiments, following the practice of the British Food Investigation Board (6), copper constantan thermocouples were employed, the individual wires of which were insulated with either shellac or celluloid, and the junctions enclosed in about a foot of glass barometer tubing of five-sixteenths of an inch external diameter. The cold junctions were immersed in a mixture of distilled water and powdered ice contained in a vacuum flask. The recording instrument was a millivoltmeter of the moving coil type made by Cambridge and Paul, and the couples were of sufficient length always to allow of the millivoltmeter being placed outside the cold chamber. All thermocouples were calibrated periodically by immersing them in (a) melting mercury and (b) in brine at various temperatures, and in which was also placed a standardized mercury-in-glass thermometer graduated into tenths of a degree.

When dealing with the larger portions of meat, e.g., hindquarters, couples were inserted at different depths, and for this purpose glass-covered couples were found to be more convenient. While it was recognized that the glass covering would be conducive to lag in the temperature recorded, it was found by comparison with bare couples that such lag was small, and in the larger portions, where freezing was a matter of hours, relatively unimportant. At a later stage of the investigations, however, when small portions were under examination, lag became of much more consequence. In order to obviate it, a bare couple with an insulation of rubber vulcanized in situ devised by Love (7) was employed. Trials of this couple showed that its lag was less than 30 seconds and that its insulation was more robust and more satisfactory than the shellac and celluloid previously used.

In each experiment the temperature was read at frequent intervals and plotted against the time. The curves so obtained were of the general form reported by the British workers (1). During freezing they flattened when a temperature of —1·5°C. had been reached, corresponding to a temperature at which a very considerable proportion of the water content of the meat solidified. From —1·5°C. the temperature fell slowly to about —3°C., after which the fall became more rapid. The curve obtained during the thawing was flattened in a similar manner. The period of time corresponding to this distinct flattening of the freezing and thawing curves is referred to as the "latent period" of freezing or thawing, as the case may be. It gives a measure of the rapidity with which freezing or thawing takes place, irrespective of the thickness of the meat or the temperature of the medium in which it is placed. In view of the many different conditions of freezing and thawing employed, this use of "latent period" was considered more suitable for purposes of comparison than a system based on the time taken for the material to pass from, say, 5°C. to —5°C.

The rates of freezing quoted throughout this paper are those found at the deepest part of the piece, i.e., the maximum latent period. No attempt was made to obtain figures when liquid air was used as the freezing medium.

(c) Estimation of "Drip."

After considerable experiment, a method was devised of obtaining an accurate comparison of the amount of "drip" by absorbing in blotting paper. Uniform slices of the thawed meat were cut about 3 mm. in thickness, they were weighed, placed between sheets of nitrogen-free, absorbent paper, and a small uniform weight placed on top to keep the meat in contact with the paper. The whole was covered by a box to reduce evaporation as much as possible. Two hours were found ample time to absorb the drip. The loss of weight of the beef was then determined, and the drip was calculated as a percentage of the weight of the beef. "Blank" drip experiments were always carried out on the fresh sample before treatment, and the difference between this value and the value for the thawed meat was called the "nett" drip. This method gave fairly consistent results; thus, in one experiment five pieces cut from the same position in the buttock gave respectively 5·45, 5.24, 5·50, 5·42, and 5·85 per cent. of drip, and in another three pieces from a rib gave 6·25, 6·50, and 6·20 per cent.

In order to obtain reliable estimations, at least three determinations were made at each position with each piece of beef under investigation.

(d) Determination of the Nitrogen Content of the "Drip."

From this determination could be calculated the approximate percentage of protein present in the drip. These determinations were carried out on the drip absorbed on the paper, by digesting the latter with sulphuric acid and estimating the nitrogen by Kjeldahl's method in the usual way.

The nitrogen loss due to irreversibility of the beef during the freezing and thawing cycle was determined by subtracting the nitrogen content of a "blank" determination on the fresh meat, from the nitrogen content of the drip obtained from the frozen and thawed meat. This nitrogen loss is expressed as a percentage of the total drip. Although it is not strictly correct to speak of this difference as the nitrogen loss, it gives a convenient method of comparison between different types of beef where blank drip contains different quantities of protein.

(e) Histological Investigations.

Microscopical examinations were made by cutting very thin sections in the frozen condition and examining them whilst still frozen. In addition, ordinary histological preparations were made after the beef had been thawed. In other experiments small pieces of frozen tissue were fixed before thawing by dropping into 10% formalin, which had been cooled to its freezing point, and sections were prepared in the usual manner. Since much distortion of structure may be caused during the usual histological preparation, more reliance was placed on the microscopical examination in the frozen state. Except where otherwise stated, the histological examinations were carried out on transverse sections from the deepest part of the frozen muscle, thus obtaining the position of maximum distortion.

As it was found that the size of the ice crystals increased concurrently with an increase in the time of freezing, measurements of the average diameters of numbers of approximately circular-shaped crystals were made. This method of measuring the amount of distortion of normal structure due to freezing, though only approximate, gave results upon which conclusions could be based. It was frequently necessary to distinguish between ice crystals and muscle fibres. For this purpose a petrological miscroscope was used, and thin sections were examined between crossed nicols. In contra-distinction to the ice crystals, the solid fibres did not extinguish uniformly, but usually showed several alternate rings of black and white.

(f) Autolysis.

Autolysis of the muscle tissue was examined by incubating the minced tissue at a definite temperature (35°C.), and estimating the percentage of the total nitrogen not precipitated by 10% tannic acid. This method is very similar to that employed by Fearon and Foster (4), except that they employed trichloracetic acid in place of tannic acid as precipitant. These workers found that by plotting the percentages of unprecipitated nitrogen against the time, in the case of fresh muscle a regular logarithmic curve was obtained, whereas with meat which had been frozen the curve became steeper, this variation increasing with the time taken in freezing. A similar result was obtained by Callow (3) with codfish muscle. These results have been explained as being probably due to the disturbance of the structure of the tissue in freezing, whereby the conditions formed on thawing become more favourable for the action of the tissue enzymes. Therefore it was considered that experiments on the rates of autolysis would yield very valuable information as to the extent of alteration of the normal structure of muscle caused by freezing.

3. EFFECTS OF RATE OF FREEZING.

Throughout these investigations, in each series quoted, beef from the same beast was employed, cut from adjacent positions on the carcase. In every case also full data were recorded as to the time between killing and freezing, age of the beast, and type of meat.

(a) On Drip and Nitrogen Content of Drip.

Table 1 shows the results of a number of experiments on pieces of a commercial size (the buttock being in the thickest portion of a side of beef), and on small pieces of standard size cut to fit the containers. In this table only those series are given which have been thawed under similar conditions, and unless otherwise stated the amount of drip and its nitrogen content refer to that from the position in the piece where it is likely to be greatest, i.e., at the position of maximum latent period of freezing. In all experiments the thawing was carried out in air.

It is seen that in every series of experiments the amount of drip steadily increases with increasing latent periods of freezing. When different series are compared there is, however, in general, very little uniformity between the quantity of drip and the latent period of freezing. As will be seen later, the type and age of the beast are of great importance in determining the degree of reconstitution of the tissue after freezing and thawing, e.g., experiments 16 and 20.

The only experiment (experiment 5) in which there was no drip was that in which liquid air was employed, and freezing was practically instantaneous. In all other experiments, even when the latent period was reduced to much less than one hour, quite an appreciable quantity of drip was found.

TABLE 1.

				Freezing.		Thawing.			
Expt.	Age and description.	Thickness Cm.	Medium.	Temp. degrees C.	Latent period hours.	Temp. deg. C.	Latent period hours.	Nett drip. %	Nitrogen loss. %
1	4 year ox buttocks	26	$CaCl_2Aq$	−36	2.1	12	40	Av. 2.2 Max. 3.1	1.02 0.93
2	„	26	Air	−10	5.5	12	40	Av. 3.7 Max. 5.4	1.10 1.17
3	—	6.5	$CaCl_2Aq$	—28	A few minutes	15	8.5	1.5	0.14
4	—	—	$CaCl_2Aq$	−12	0.75	15	8.5	3.0	1.00
5	—	4	Liq. Air	—193	A few seconds	15	—	−0.1	0.12
6	—	4	Air	—10	10	15	—	2.5	0.26
7	6 year ox pieces	4.5	$CaCl_2Aq$	−18	0.5	5	18	0.7	0.30
8	„	6.25	$CaCl_2Aq$	—18	0.75	5	22	3.6	0.35
9	6 year ox pieces	3.0	$CaCl_2Aq$	—18	0.4	19	1.75	2.9	0.25
10	„	4.5	$CaCl_2Aq$	−18	0.5	19	2.25	3.7	0.45
11	„	7.5	$CaCl_2Aq$	−18	1.1	19	5.0	7.8	0.47
12	„	8.5	$CaCl_2Aq$	−18	1.7	19	6.0	5.5	0.26
13	aged ox pieces	4.8	$CaCl_2Aq$	10.5	1.55	6	21	3.6	0.30
14	„	6.7	$CaCl_2Aq$	—10.5	2.30	6	36	3.7	0.38
15	„	8.5	$CaCl_2Aq$	—10.5	4.00	6	39	4.3	0.47
16	4-5 year ox pieces	3.3	$CaCl_2Aq$	—14	0.6	1	25	1.4	0.26
17	„	5.0	$CaCl_2Aq$	—14	1.1	1	50	1.5	0.37
18	„	7.0	$CaCl_2Aq$	−14	2.2	1	64	3.4	0.13
19	„	9.5	$CaCl_2Aq$	−14	5.0 (?)	1	96	3.9	0.16
20	very aged ox pieces	3.3	$CaCl_2Aq$	—23 to —27	0.18 0.55	0.6 0.6 to .20	65 64	3.1 4.4	0.15 0.19
21	„	5.0	$CaCl_2Aq$	—23 to −27					
22	„	7.0	$CaCl_2Aq$	—23 to —27	1.70	0.6 to .20	88	6.6	0.19
23	„	10.0	$CaCl_2Aq$	23 to —27	2.30	0.6 to .20	99	7.4	0.28

The nitrogen loss figures show that the protein loss usually ran parallel with the increase in latent period, but there was not the same regularity as with the drip.

(b) Histological Structure.

Photomicrographs of frozen sections were taken (see plates, figures 1-13), but it proved very difficult to cut sections which contained ice, and which were sufficiently thin and uniform to eliminate the effect of depth, and consequently some of the sections are slightly out of focus in several places.

Figure 1 represents a section of beef frozen by means of liquid air. In this case, owing to the opacity of the section, individual fibres can only be distinguished with difficulty, but no ice crystal formation is visible.

Figures 2-7 represent a series in Table 1, experiments 9-12, and show a gradual increase of distortion of the tissue with increasing latent period of freezing.

Experiment 9 (latent period of freezing 0·4 hour) (figure 2) shows an unaltered structure as compared with that of fresh beef, save for the beginning of ice crystal formation in a few places, such as at B. The fibres are regularly dispersed, and of uniform polygonal shape, as in a normal section. It is evident that this figure illustrates the beginning of definite distortion, since there is a very small amount of ice formation outside the fibres, and in several places the latter appear to have become compressed, owing to the small amount of ice crystallized around them, forcing them together. Figure 3 is from the same piece, but has been fixed whilst in the frozen condition. Its structure is very similar to that of the frozen section, except that in a large number of the fibres there appears a small hole, as at A, which in the frozen condition has probably been a rod of ice inside the fibre. This demonstrates that the alteration of the normal colloidal structure can definitely take place not only outside, but also inside the fibre, and that the two types of distortion can go on simultaneously. Also since this experiment shows a much greater amount of ice formation within the individual fibre than without, it would appear that the alteration of structure due to freezing commences within the fibre itself.

Figures 4 and 5 are sections, the one in the frozen state and the other after fixing and staining, of the beef frozen in experiment 10 (latent period 0·5 hour). They show that with a slight increase in the time of freezing the alteration of the normal structure has increased. It is difficult to distinguish the ice crystals in figure 4, but figure 5 definitely shows that ice has formed, as at A, in small numerous crystals outside the fibres. The amount of ice formation is, however, not great. In most cases the muscle fibres are regular, but many have been laminated by the pressure of the ice crystals. The freezing has evidently been slow enough to allow of much water separation from the plasma, and its passage into the surrounding medium, since many of the fibres are very small, and the number which show cavities left by ice crystals inside are fewer than in experiment 9.

Experiment 11 (latent period 0·75 hour), figure 6 shows a further stage in the distortion of structure. The amount of ice formation, as at A, and the size of the ice crystals, show that the "dehydration" of the fibres has had time to proceed further. From the presence of so many intact fibres, as at B, it is evident that the freezing has been fast enough to prevent a large percentage of the fibres from becoming both "dehydrated" and distorted by the pressure of the

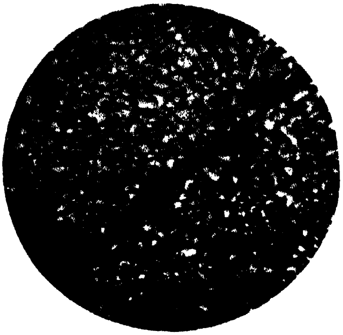


Fig. 1.

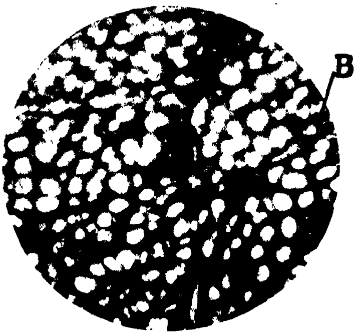


Fig. 2.

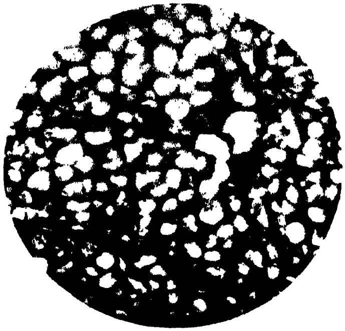


Fig. 4.

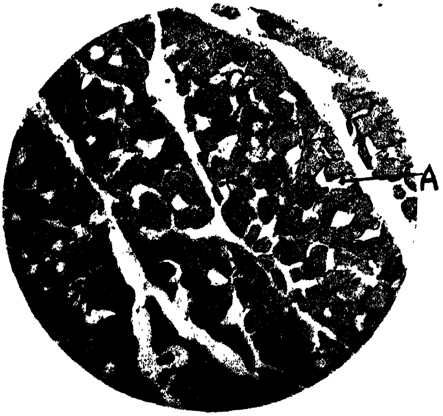


Fig. 5.

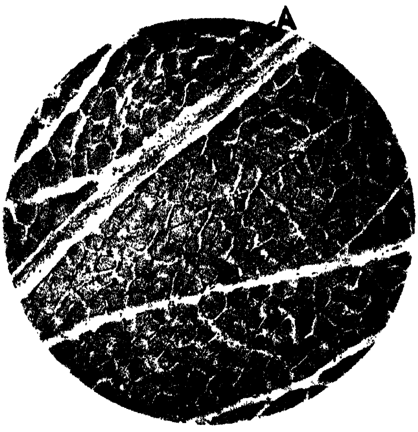


Fig. 3.

PLATE 1. SECTIONS OF FROZEN BEEF.

Figs. 1, 2, 4 show sections in the frozen condition.

Figs. 3, 5 show sections which have been fixed whilst frozen, and stained with eosin and haematoxylin.

ice crystals. In this piece also another result of distortion appears, viz., the forcing together of muscle fibres, connective tissue, etc., into a compressed mass by two or more sets of ice crystals which have grown towards one another. Such bands of compressed matter may be seen at C. That this compressed mass has lost a large percentage of its original water content may be judged from the fact that the thin bands are very dense and yellow in colour by transmitted light, and quite unlike the clear or whitish appearance of the undistorted frozen muscle fibres.

Whereas in the last experiment the ice crystals occupy an area of about one-third of the cross section, in figure 7, experiment 12 (latent period 1·7 hours) they occupy at least one-half. It is also seen that the bands of compressed material are more numerous and longer and wider than before.

Similar sections were obtained from other experiments on small pieces of beef recorded in Table 1. It was generally observed that as the latent period of freezing increased the degree of distortion became more marked. There was, however, a certain want of uniformity in the degree of distortion produced in samples of different origin, which differences were probably due to the different ages of the beasts, and also possibly to being reduced to different final temperatures during the freezing. In no case, however, was the original structure preserved after freezing.

Figures 8, 9, and 10 refer to experiments in which pieces were frozen in brine and air, latent periods of freezing being 2 and 10 hours respectively. Figure 8 illustrates the structure of the piece frozen in brine; figure 9 a section of the air-frozen piece in the frozen condition; and figure 10 the same after fixing.

In figure 8 the ice occupies about one-third of the total area, occurring mainly as small isolated crystals. In figure 9, however, it occupies nearly one-half of the total area, and the crystals are relatively large (up to 0·4 mm. width), as may be seen at A in both figures. Another striking point of difference is that there is quite a considerable number of isolated intact fibres present in figure 8, whilst in figures 9 and 10 there are very few. In the former case a certain number only of "dehydrated" and distorted fibres are collected into compressed bands, whereas in the latter almost all the fibres are so arranged (B). These are usually of much smaller area in cross-section, and are laminated in the direction of the compressing ice crystals.

Figures 11 and 12 are taken from the large scale pieces (buttocks) in experiments 1 and 2, Table 1. As it was found impracticable to obtain sections from the centre of the buttocks, wedge-shaped pieces were cut from similar positions in each, and sections were made from these. It was estimated that the approximate latent periods of freezing of these sections were 1 and 20 hours respectively. The difference in appearance is very striking. In figure 11, although the uniform structure has been altered, the number of undistorted fibres is fairly large, the size of the bands of compressed fibres very small, and the quantity of ice formation not extensive. In figure 12 all resemblance to the structure of mammalian muscle has disappeared. It shows large ice crystals and numerous bands of compressed fibres, which have lost so much water that they appear as dense opaque masses, even in the thinnest sections. The jagged appearance of the ice crystals is due to the difficulty of cutting material containing so much ice without causing the surface of the crystals to become uneven. In no case was such a rate of freezing attained with large pieces of beef that alteration of tissue structure disappeared, the structures presented in the sections from experiment 1 showing the least amount of distortion of any of these experiments.

An attempt was made to compare the size of the crystals of ice. A rough method was employed, which consisted in estimating the average diameters of all ice crystals approximately circular or elliptical in cross-section. A micrometer eye-piece of the rotating type was used capable of measuring to at least ±0·2μ, and in each case the average was obtained from measurements of twenty-five to thirty crystals.

The figures for the experiments of Table 1 are collected in Table 2, and, with one exception, show an increase in the size of the ice crystals with increasing latent period of freezing.

TABLE 2.

Experiment.	Latent period of freezing hours.	Average diameter of ice crystals.
1	2.1	117
2	.55	231
7	0.5	101
8	0.75	134
12	1.70	175
13	1.55	169
14	2.30	159
15	4.00	204
16	0.6	128
17	1.1	144
18	2.2	145
19	5.0	176
20	0.18	109
21	0.55	116
22	1.70	139
23	2.30	152

The general results of the histological investigation show that the only case in which a normal structure was retained after subjecting the tissue to freezing

PLATE 2. SECTIONS OF FROZEN BEEF.

Figs. 6, 7, 8, 9 show sections in the frozen condition.

Fig. 10 shows a section which has been fixed whilst frozen and stained with eosin and haematoxylin.

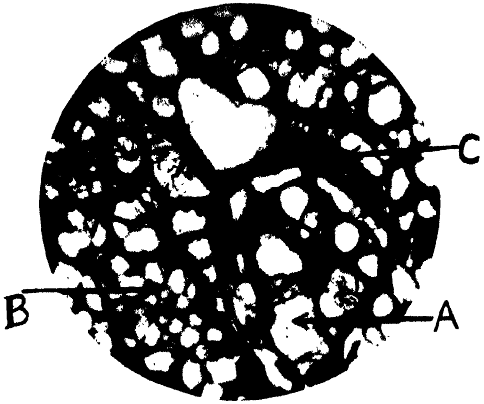


Fig. 6.

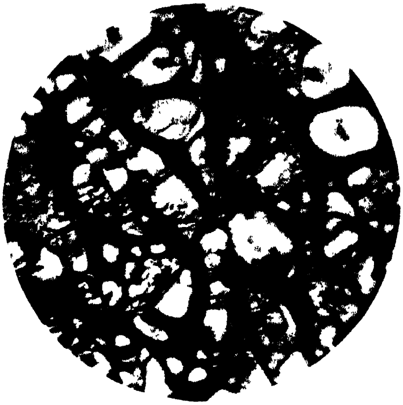


Fig. 7.

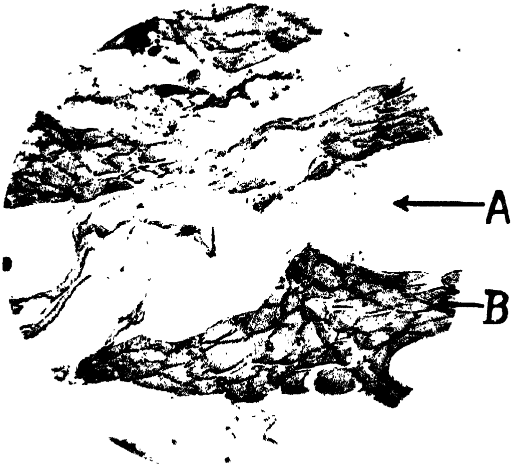
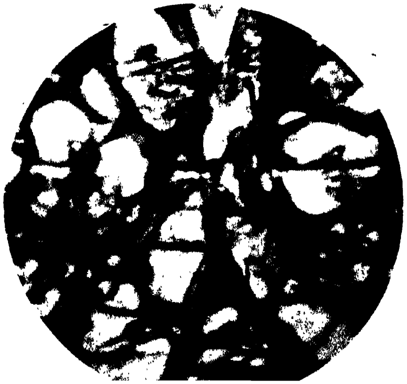
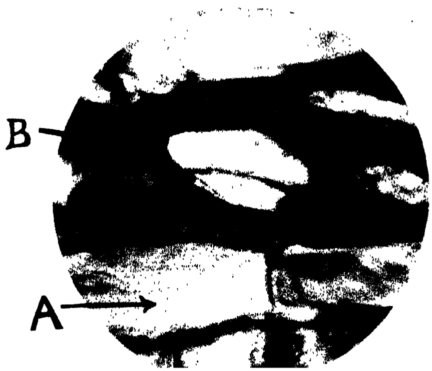
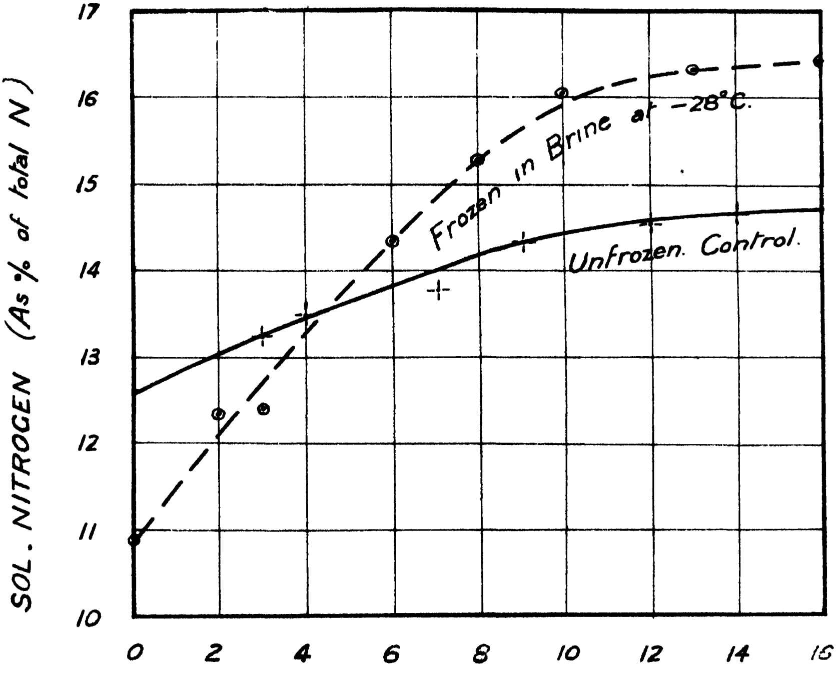


Fig. 10.





was in the piece frozen almost instantaneously in liquid air. The structure most closely resembling this was that shown in figure 9, latent period 0·4 hour, where alteration of the normal colloidal condition had proceeded to some extent inside the fibre, but where there was very little ice formation outside. In all the other pieces, even that (experiment 20) having a latent period of only 0·18 hours, definite distortion of structure was seen. These results were borne out by the figures for the measurement of the ice crystals.



Autolysis of Beef at 35° C.

Two other phenomena which accompany an increase in the latent period of freezing are the gradual lessening of the numbers of distorted, clear fibres, and the increase in the width and length of the bands of compressed "dehydrated" fibres.

(c) *Rate of Autolysis.*

A typical experiment on the comparative rates of Autolysis in fresh and treated beef is shown in the accompanying curves (A and B). A was obtained from a sample from a buttock which had been frozen in brine at —25°C. It was cut from a position at a depth of 6 cm., and had a latent period of 1½ hours. B was from the control with unfrozen beef from the same buttock.

It will be noticed that the forms of these autolysis curves are very different. In the case of the frozen beef, both the rate and extent of autolysis is greater. The sample which has been frozen has an initially lower percentage of soluble nitrogen than the fresh meat, usually amounting to about 2%. No explanation has been found for this lowering of the soluble nitrogen content. As suggested by Fearon and Foster (4), the differences in the forms of the autolysis curves are probably due to an alteration produced in the colloidal structure by freezing, which causes it to be more easily hydrolyzed by the autolytic enzymes of the tissue.

4. EFFECT ON THE RATE OF THAWING.

(a) On Drip and Nitrogen Content of Drip.

Previous experiments on the thawing of frozen gels (see introduction) had shown that a considerable quantity of water which had separated during freezing was reabsorbed when the frozen gel was thawed slowly. It might be expected, therefore, that water which had separated from the colloids of beef tissue during freezing might also be reabsorbed, and the amount of drip consequently reduced, if a slow rate of thawing were employed.

As the result of histological examinations, Kallert (5, 6), and Richardson and Scherubel (12), have stated that frozen meat after slow thawing exhibits less change than after rapid thawing. These workers, however, made no quantitative observations on the amount of drip produced, so that in the present investigation it was thought advisable to carry out such determinations, using different rates of thawing. As before, experiments were carried out both on small pieces of beef and on whole buttocks, and in each comparative series the freezing was carried out in exactly the same manner. The drip was estimated as soon as possible after thawing, so as to eliminate the possibility of subsequent absorption. The results are given in Table 3, the freezing medium being calcium chloride solution throughout.

TABLE 3.

			Freezing.		Thawing.				
Expt.	Age & description.	Thickness. Cm.	Temp. deg. C.	Latent period hours.	Medium	Temp. deg. C.	Latent period hours.	Nett drip. %	Nitrogen loss. %
7	6 year ox	4.5	—18	0.5	air	5	18.0	0.7	0.30
10	pieces	4.5	—18	0.5	air	19	2.25	3.7	0.45
24	aged ox	8.0	—15	2.0	air	0.6	90	0.8	0.08
25	pieces	8.0	—15	2.0	air	20.0	4	1.6	0.56
26	aged cow	21	—25	2.8	air	10	54	1.7	0.21
27	buttocks	21	—25	2.8	water	15	14 (?)	3.4	0.47

Fig. 11.

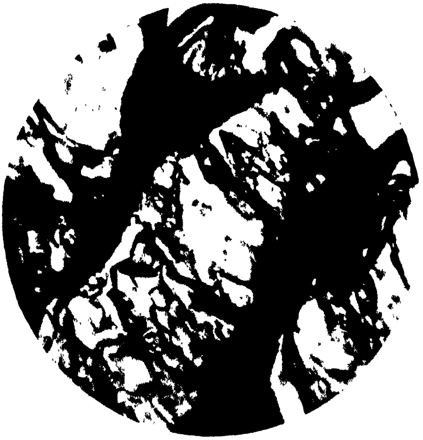


Fig. 12.

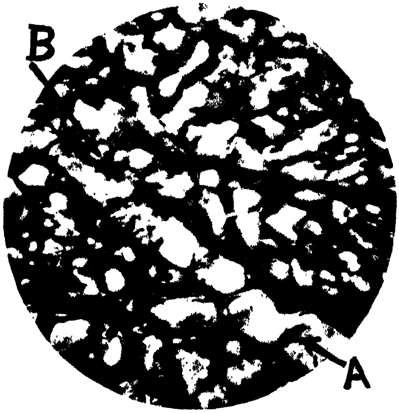


Fig. 13.

PLATE 3. SECTIONS OF FROZEN BEEF AND MUTTON.

Figs. 11, 12, 13 show sections in the frozen condition.

These experiments show that slow thawing had reduced the drip, but that in no case had the drip been completely eliminated.

5. EFFECT OF STORAGE AFTER THE COMPLETION OF THAWING.

Experiments were also performed to ascertain whether the tissue, after thawing had been completed, would still continue to absorb the water separated during freezing. These experiments were carried out by storing the pieces at a temperature above their freezing point, viz., at 1°C. A series of such experiments is given in Table 4. The material used throughout was aged ox, and the freezing medium was calcium chloride solution. The thawing temperature varied from 1° to 20°C.

TABLE 4.

Expt.	Time of storage at 1° C. after thawing. days.	Thickness. Cm.	Freezing. Temp. deg. C.	Freezing. Latent period hours.	Thawing. Latent period hours.	Nett drip. %	Nett nitrogen loss. %
20	0	3.3	−23 to −27	0.18	65	3.1	0.15
28	7	3.3	−23 to −27	0.18	64	5.1	0.17
21	0	5.0	−23 to −27	0.55	64	4.4	0.19
29	7	5.0	−23 to −27	0.55	65	3.3	0.10
22	0	7.0	−23 to −27	1.70	88	6.6	0.19
30	7	7.0	−23 to −27	1.70	88	5.7	0.16
23	0	10.0	−23 to −27	2.30	99	7.4	0.28
31	7	10.0	−23 to −27	2.30	99	3.2	0.11

In three cases out of four, there was thus less drip after storage than immediately after the completion of thawing.

6. EFFECT OF STORAGE AT 1°C. BEFORE FREEZING.

As it had been pointed out in correspondence with the British Food Investigation Board that the osmotic pressure of the contents of the muscle fibres rises steadily after the death of the animal, experiments were carried out to see whether this rise of osmotic pressure had any effect on the amount of drip liberated from a muscle tissue on thawing. It was believed possible that this rise might facilitate in the imbibition by the fibres during thawing of the water liberated outside them.

In order to test this suggestion two series of experiments were carried out by comparing the amount of drip and its nitrogen content from pieces of beef of equal thickness cut from the same carcase, and subjected to the same conditions of freezing and thawing, except that each piece had been stored at a temperature of 1°C. for a variable length of time before freezing. In this way a series of seven similar samples could be frozen with a definite interval of time between each freezing. In the second set of experiments the rate of autolysis (and perhaps putrefaction) of the stored beef was followed in order to determine whether any extensive changes had taken place in the chemical composition of the beef. In this series the first piece to be frozen was a cut from a beast immediately after killing. Every effort was made to prevent putrefaction from taking place in the stored samples by sterilizing the containers and the sheet rubber coverings which were placed on each container to prevent drying out of the tissue.

The results are given in Table 5, the freezing and thawing media being calcium chloride and air respectively.

TABLE 5.

				Freezing.		Thawing.			
Expt.	Length of time between killing and freezing.	Age and description.	Thickness. Cm.	Temp. deg. C.	Latent period hours.	Temp. deg. C.	Latent period hours.	Nett drip %	Nett nitrogen loss %
17 (a)	4 days	5 year ox	5·0	—12·5	1·2	1	55	1·5	0·37
17 (b)	11 days	5 year ox	5·0	—12·5	1·2	1	55	0·9	0·11
17 (c)	18 days	5 year ox	5·0	—12·5	1·2	1	55	2·95	0·16
32	2 hours	aged bull	5·0	—17	1·0	4	15	5·5	—
33	5 days	aged bull	5·0	—17	1·0	4	15	4·7	—
34	10 days	aged bull	5·0	—17	1·0	4	15	2·8	0·71
35	15 days	aged bull	5·0	—17	1·0	4	15	4·9	0·71
36	20 days	aged bull	5·0	—17	1·0	4	15	2·2	0·85

The results given in Table 5 show that up to the end of ten or eleven days' storage at a temperature of 1°C. before freezing, there is a definite decrease in the drip from the beef after freezing and thawing. When stored for a longer period a slight increase is observed. Experiments carried out on the extent of autolysis show only a slight increase (approximately 10%) of unprecipitated nitrogen, even after twenty days' storage.

7. EFFECT OF AGE OF MUSCLE.

The experiments already given in Table 1 show that generally speaking there was much less drip with the muscle tissue of younger animals than with that of older beasts, and the histological examination also showed much less ice formation, and in consequence less distortion of structure than with the older muscle. Table 6 gives the results of experiments bearing directly on this question, carried out by freezing beef of different ages, each piece being subjected to the same conditions of freezing and thawing. The samples were frozen in calcium chloride solution at —10·5°C., in containers of 4·8 cm. width, and were thawed in air at 6°C. The latent periods of freezing and thawing were 1½ hours and 21 hours respectively.

TABLE 6.

Experiment.	Age and description.	Nett drip, %	Nett nitrogen loss, %
13 (a)	1½ years ox	2·6	0·33
13 (b)	4 years ox	3·5	0·33
13 (c)	very aged ox	3·6	0·30

The figures show a lower amount of drip in the younger muscle than in the older.

Histological examination showed that there was a moderate amount of structural alteration in each tissue, but that the size and amount of ice crystals which had separated out was less in the youngest than in the four years old and the aged muscle.

8. COMPARISON OF BEEF AND MUTTON.

Mutton differs from beef in that when it is frozen on the commercial scale in air at —14°C. ("sharp" freezing), almost complete reversibility takes place on thawing. It was therefore thought possible that a comparison of the changes in beef and mutton brought about by freezing might yield information as to the irreversibility of beef on slow freezing.

Two similar samples of mutton were frozen, the one in liquid air (latent period of a few seconds), and the other in air at —14°C. (latent period approximately 10 hours). Sections were taken from the deepest parts and examined in the frozen condition. Owing to the opacity of the former the cutting of suitable sections proved very difficult, but no distortion of the normal structure could be observed. A section cut from the slowly frozen piece is seen in figure 13. There is considerable ice crystal formation, but most of the crystals are small (A), whilst many intact, undistorted fibres may be seen (B). It is interesting to compare this with sections of beef frozen with approximately the same latent period (figures 9 and 10). The differences in the two types of tissue are at once apparent, even when allowances are made for the slightly smaller magnification in the case of mutton. The distortion in the mutton section is practically the same as that shown by the beef illustrated in figure 6, and having a very short latent period of freezing (0·75 hour). Mutton is thus much more resistant to slow freezing. The reason for this difference is at present unknown.

SUMMARY AND DISCUSSION.

In the experiments recounted in this paper it was found that:

(1) Increasing the latent period of freezing increased the changes in the beef, as determined by the quantity of drip, the nett nitrogen loss on thawing, and by the microscopic appearance in the frozen condition. In the frozen state, when the latent period of freezing was longer, the ice crystals were observed in smaller

numbers, but of larger size, whilst there were more bands of compressed "dehydrated" fibres, and fewer undistorted fibres than in the rapidly frozen samples. The disorganization of the muscle structure was also shown by the increase in the rate and in the extent of autolysis in the material after thawing.

(2) Marked differences were noted between the muscle tissue from animals of different age; in general, younger muscle could be frozen and thawed with less change than that from older beasts.

(3) The degree of reconstitution of the colloidal material was increased by increasing the latent period of thawing, since the quantity of drip was materially reduced when the frozen meat was thawed slowly. It could also be reduced by storage at 1°C. after thawing, showing that the imbibition of liquid by the colloid still went on after thawing was complete.

(4) In general the drip was less when the material was stored for a length of time not exceeding ten days at 1°C. before freezing, which may possibly be due to increased osmotic pressure in the muscle content.

(5) When frozen under similar conditions mutton showed much less ice formation and distortion of structure than beef.

The only experiments in which complete reversibility occurred, i.e., where there was no drip after thawing, and no apparent distortion of the frozen structure, were those in which liquid air was employed, giving an extremely rapid rate of freezing. The next best was obtained with a small piece by using a calcium chloride medium. This piece had a latent period of freezing of one-half hour. In this there was very little drip, and very little visible ice formation. To obtain such a rate with pieces of a size actually used in practice, such as quarters of beef, it would be necessary to use some freezing medium of approximately the same specific conductivity as saturated calcium chloride solution, and at a temperature considerably lower than —60°C. In addition to the mechanical difficulties involved, this would, according to Plank (10) introduce another factor in producing irreversibility, viz., that of low temperatures. Plank believes that some of the water in the muscle colloid is more closely bound to the colloid than the remainder, and that this bound water does not completely freeze out until a very low temperature is reached, viz., —56°C. When once frozen out, however, it is not readily reabsorbed during thawing. According to this view there is, therefore, a critical temperature below which this effect occurs. Some of the results quoted in Table 1 may possibly be attributed to this effect of low temperature. In experiments 14 and 23, for example, the same type of beef was employed, and it was frozen by means of a calcium chloride bath, in the former case at —10·5°C., and in the latter at —23° to —27°C. Both had approximately equal latent periods of freezing; yet the drip was considerably greater where the lower temperature was employed, even though there was a slower rate of thawing.

The conditions tested in this investigation which were found to affect the quantity of drip, and the structural alteration, were therefore the rate of freezing, the rate of thawing, the time of storage after thawing, storage before freezing, and possibly the temperature to which the meat was reduced in the freezing process. Of these factors the first appears to be the most important.

The work, however, indicates that though many factors are involved, it may yet be possible to find a combination of conditions by which beef may pass through the refrigeration cycle with the minimum amount of change.

The authors desire to acknowledge the help afforded them by the A.N.R.C. Committee previously referred to, and also their appreciation of the very active interest taken by Mr. J. Hepburn, Works Manager and Chief Engineer, Victorian Government Cool Stores, who made the arrangements for the necessary refrigerating facilities. The investigation was facilitated by the generous co-operation of the Victorian Agricultural Department and the Institute of Science and Industry, and the University of Melbourne.

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BLOOD-GROUPING OF AUSTRALIAN ABORIGINALS.

by

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(Submitted for publication 9th February, 1926.)

The Australian aboriginal is rapidly dying out. Comparatively few pure-blooded natives are now met with in the settled districts. In the central and northern parts of the continent their numbers are, however, still considerable.

The Australian native has apparently been almost completely, if not for practical purposes absolutely completely, isolated from other races of mankind for a period to be measured in tens of thousands of years. As remains of the dingo, his presumed companion when he arrived in Australia, are said to have been found in association with larva deposits, it is possible that he may have been in Australia when the extinct volcanoes in the Mount Gambier and Warrnambool district were in eruption. The other races of mankind have had frequent chances of mixing with each other, so that hybridization must have taken place to a greater or lesser extent.

From the results obtained in the blood-grouping of these various races there is a possible suggestion that they are composed of varying intermixtures of two sub-species of man. In the European at one end of the scale, one of these sub-species may exercise a more dominating influence, as shown in the blood-grouping, than is the case, say, in negroes or Hindus, towards the other end of the scale. This raises the question as to whether the Australian aboriginal may have been segregated at such an early stage that the intermixture of the two sub-species had not yet taken place, with the result that only one agglutinogen might be met with in an examination of individuals of this race. Under any circumstances, it was advisable to make a blood-grouping of the aboriginals, to place on record, before they disappeared, the relative proportions of the different groups met with in as large a number of individuals as possible.

During the last few years every opportunity has been taken, when making journeys in the back country, to ascertain the blood-groups of any pure-blooded natives met with. The result is that up to date 101 of such examinations have been made, and only two blood-groups have been met with—55 of the individuals belonging to Group II, and 46 to Group IV. It is the purpose of this short communication to place on record the results so far obtained.

When travelling in the interior it is often a difficult matter, especially in the open, to carry out successfully and in full detail all the tests that might be made. It is only found practicable, in the majority of cases, to take Type Sera II and III for testing purposes. After little, or sometimes much, persuasion, and the presentation of tobacco or sweets, and an explanation of the purpose in view presented in pidgin English, it has been possible to obtain the consent of individuals. A drop of blood was then drawn into a little citrate solution, and finally mixed with the respective sera on a glass slide by means of the end of a match. On rocking the slide clear-cut results were obtained.

Point McLeay Natives. With the assistance of Dr. Barlow, in 1922, 16 natives at Point McLeay, on Lake Alexandrina, were tested; 3 belonged to Group II and 13 to Group IV. Of the males, 3 belonged to Group II and 6 to Group IV. Of the females, 0 belonged to Group II, and 7 belonged to Group IV.

North-East of South Australia. On a visit in May, 1924, to the extreme North-east portion of South Australia, blood-groupings were obtained at Murnpeowie, Innamincka, Cordillo, and Arrabury, just over the Queensland border. Sixteen belonged to Group II and 12 to Group IV. Of the males, 5 belonged to Group II and 5 to Group IV. Of the females, 11 belonged to Group II and 7 to Group IV.

Tarcoola District. In November, 1925, Dr. Clark Wissler and Mr. E. Embree, representatives of the Rockefeller Foundation, were taken on a visit to Wilgena, near Tarcoola. Blood-groupings were made on this expedition of 55 of the natives, with the assistance of Professor Brailsford Robertson; 36 belonged to Group II and 19 to Group IV. Of the adult males 18 belonged to Group II and 8 to Group IV; and of the adult females 12 belonged to Group II and 8 to Group IV. Of the children of mixed sexes 6 belonged to Group II and 3 to Group IV. During the examination of these natives close observation was made of their physiognomy by Dr. Clark Wissler, Mr. Embree, and Professor Robertson, and these observers were under the impression that they could frequently foretell to what particular group an individual native would be found to belong. This, if correct, is exceedingly important, but will require much fuller investigation.

At the Adelaide Hospital, in 1923, one native, tested by Dr. Barlow, was found to belong to Group IV. Another, a member of the famous Arunta tribe, also belonged to Group IV.

The results may be shown in tabular form as follows:

	Group II.	Group IV.	Total.
	(Moss's Classification)		
Point McLeay	3	13	16
N.E. of South Australia	16	12	28
Tarcoola District	36	19	55
Adelaide Hospital	0	2	2
	55	46	101

It will be seen that the relative proportions of these two groups vary somewhat in the different districts. In the southernmost (Point McLeay) there are only 3 of Group II and 13 of Group IV. In the N.E. of South Australia Group II slightly exceeds Group IV, and amongst the Tarcoola blacks Group II is nearly twice as numerous as Group IV. When the latter (the Tarcoola blacks) are divided further into males, females, and children, we find that the same relative proportion exists. The examinations made at Cordillo included a mother aged about forty and her twin daughters, aged seventeen; these all belonged to Group IV.

It thus appears that amongst the small number of natives (101) thus far examined by us in Southern Australia no member of Group I or Group III (Moss's Classification) has been met with. This part of Australia is considerably remote from the northern and north-eastern portions, where it is possible that recent admixtures with Malays and Papuans may have occurred.

Dr. A. H. Tebbutt (1, 2) has carried out a number of examinations on presumably full-blood Australian natives in New South Wales and Queensland. He has combined his results in the second of these two papers. He tested a total of 191, of which 14 were in New South Wales and 177 in Queensland. Transposing his groups from the Jansky to the Moss Classification, he found amongst the New South Wales natives 1 in Group I, 7 in Group II, 0 in Group III, and 6 in Group IV. Amongst the Queensland natives he found 1 in Group I, 66 in Group II, 11 in Group III, and 99 in Group IV. He thus met with 2 examples of Group I and 11 examples of Group III amongst 191 natives. It would thus seem as though all four groups are to be met with amongst pure-blooded Australian aborigines, unless the presence of Groups I and III is to be explained by a recent and slight intermixture with a foreign race coming from the north. In any case, our results show that the Australian native differs from all other races in having a biochemical index (iso-agglutinin index), as already pointed out by Dr. Tebbutt, higher than any other, and still higher than the English, which otherwise tops the scale. According to the blood-grouping the Australian native is ultra-European.

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THE EFFECT OF THYROID FEEDING ON THE OESTROUS CYCLE OF THE GUINEA PIG AND MOUSE.

by

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(Submitted for publication 12th February, 1926.)

The relationship between the activity of the thyroid gland and the function of the ovaries in the human female is attested by a large amount of clinical evidence. The enlargement of the thyroid sometimes seen during menstruation and pregnancy, the occurrence of thyroid disorders at puberty and the climacteric, the delayed development of the genital organs in cretins, and the disturbances of menstrual function which frequently accompany thyroid disease, suggest such a relationship, though the profound disturbances of general metabolism which accompany alterations in the secretory activity of the thyroid may well account for all the observed changes in genital function.

The experimental results of thyroid feeding and of removal of the thyroid gland in animals are less clear. Hammett (9) showed that removal of the thyroid apparatus in rats 45 to 56 days old did not inhibit sexual activity or prevent reproduction, though breeding was delayed and fewer and smaller young were produced. In sexually mature albino rats, 100 to 150 days old, removal of the thyroid and parathyroid caused retrogression of the ovaries and uterus, while removal of the parathyroid alone had little effect. Alquier and Theuveny (4), using adult dogs, and Bensen (6), working with rabbits, found no alteration in ovarian structure after removal of the thyroid apparatus, though Hofmeister (12) had found evidence of follicular degeneration of the ovaries of rabbits after thyroidectomy. Evans and Long (14), using rats of various ages, found that thyroidectomy resulted in cessation of oestrus for from 6 to 27 days, after which the oestrus occurred normally. In young animals there was no effect on the time of maturity or on the length of the oestrus cycle, though in these, evidences of regeneration of thyroid tissue were found at autopsy, which may have explained the irregular results.

Herring (11) found that thyroid feeding in white rats caused an acceleration of the growth of the ovary, and Evans and Long (14) observed a prolongation of the oestrous cycle or inhibition of oestrus when rats were fed with large doses of ox thyroid, though with small doses, sufficient to cause loss of weight and increased consumption of food, the oestrous cycles were not greatly disturbed.

In the present paper we have studied changes in the oestrous cycle of the guinea-pig and mouse, using a dosage of thyroid graded according to the weight of the experimental animal.

Methods.

The demonstration by Stockard and Papanicolaou (16) of a regularly recurring cycle of changes in the microscopical appearance of the vaginal secretion of the guinea-pig, correlated with structural alterations in the lining of the genital tract, has afforded a means of accurately determining the onset of oestrus and the duration of the oestrous cycle. The method has been found to be applicable in the pig (7), rat (14), mouse (1), opossum (10), rabbit (17), and in the cow (15). It is possible, also, by the vaginal smear method, to study the effect of various procedures on the function of the reproductive system, for it is reasonable to suppose that any marked disturbance will be reflected in the oestrous cycle.

Our work was done on virgin female white mice, three months old, of which ten were fed with thyroid extract, while five served as controls, the whole group of animals being kept under identical conditions and fed on bread and milk, sunflower seeds, and whole maize. Our guinea-pig experiments were carried out on eighteen animals, varying in weight between 420 and 708 grams, of which twelve were non-pregnant females, which were segregated with their offspring immediately after parturition, the young being removed when three weeks old, while six were virgin females. Eight of the non-pregnant but parous females were fed with thyroid, and the remaining four, together with the six virgin females, were used as controls. All the animals were fed on a diet of cabbage, carrot, and bran, and were kept under similar conditions.

The guinea-pig experiments extended over a period of 64 to 113 days, while the mice were observed for 240 days.

Vaginal smears were made daily throughout the experiments. These were easily obtained by the use of a small piece of cotton wool tightly wound round fine forceps, and inserted high up into the vaginal canal. Smears were stained for two minutes with undiluted Leishman's stain and subsequently washed with tap water. This stain gives a more brilliant picture than the methylene blue used by earlier workers. Leucocytes show a deep purple nucleus, cornified vaginal cells are unstained, whilst recently desquamated vaginal and uterine cells show faint pink cytoplasm with blue nuclei. Figures 1 to 4 illustrate very well the changes occurring in the vaginal secretion during the oestrous cycle of the mouse.

Throughout the period of thyroid feeding we have employed carefully graded

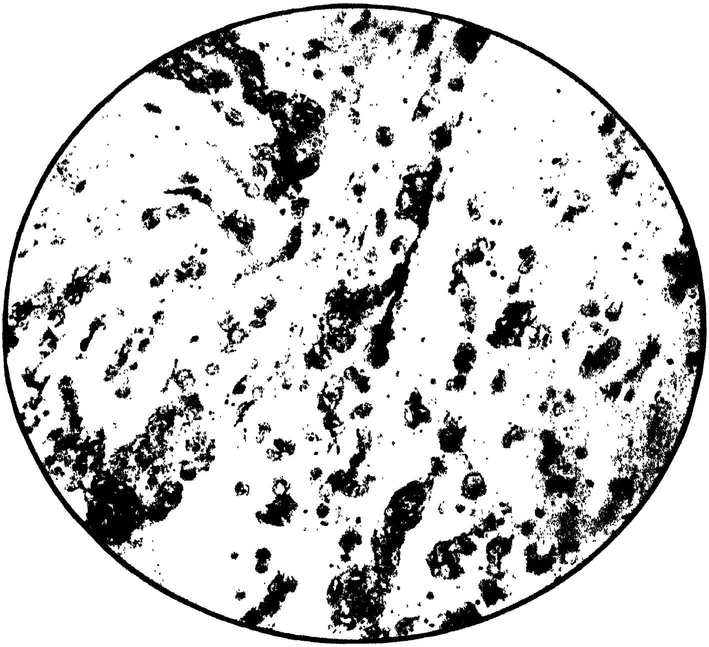

Fig. 1. Magnification 72.

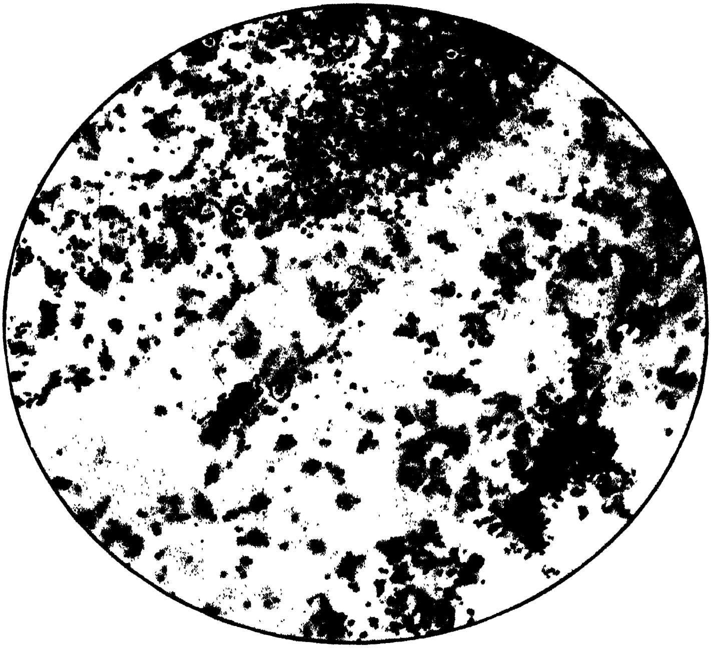

Fig. 2. Magnification 72.

doses of thyroid extract. Burroughs, Welcome's preparation, containing not less than 0·05 per cent. of iodine, was used. A tablet containing 0·002 grams of dried thyroid was specially prepared for us by this firm, so that we were able to employ a dosage of one forty-thousandth to one ten-thousandth of the body weight. We usually commenced with the smaller dose, and gradually increased it. No difficulty was experienced in feeding the guinea-pigs, but with mice we had some trouble until we devised the plan of grinding the thyroid extract up with a small quantity of condensed milk, which the animals took readily. The potency of this preparation was shown by the occurrence of the classical signs of experimental hyperthyroidism in both species of animals—loss of weight, falling out of hair, increase of heart rate, transient attacks of diarrhoea, with loss of strength and diminished activity. Occasionally exophthalmos was noted in the guinea-pigs.

TABLE I

Weight in grams	Number of days observed	Duration of cycles in days	Average cycle in days	Duration of oestrus in days	Average oestrus in days
		CONTROLS			
545	64	16 16 16	16	2 2 1	1·7
470	64	16 17 16	16·3	1 2 2	1·7
532	64	16 17 17 18	17	1 2 2 1	1·5
420	64	16 17	16·5	2 2	2
GROUP AVERAGE			16·5		1·73
320	113	17 16 15 15 16 16	15·8	1 1 2 1 1 2	1·3
355	113	18 16 15 16 15 17 16	16·1	1 1 2 2 2 1 2	1·5
340	113	17 16 15 15 15 15	15·5	1 2 2 1 2 2	1·7
295	113	17 15 16 15 15 15 16	15·5	1 1 1 1 2 2 2	1·4
425	113	17 16 15 15 16	15·8	1 2 2 2 2	1·8
365	113	16 17 15 15 15 15	15·5	2 1 2 2 2 2	1·8
GROUP AVERAGE			15·7		1·6
		THYROID FED			
500	64	16 16 17	16·3	3 4 5	4
547	64	15 16 17	16	4 4 5	4·3
640	64	15 15 16 17	15·75	4 5 5 5	4·8
422	64	16 17	16·5	4 5	4·5
708	64	16 17 17	16·6	5 5 6	5·3
477	64	15 16	15·5	5 5	5
620	64	15 16 16 16	15·75	4 4 5 5	4·5
485	64	16 17 16	16·3	4 5 6	5
GROUP AVERAGE			16·1		4·7

Results.

Our observations on guinea-pigs are set out in Table 1. Of the controls, the first four are non-pregnant parous females, and the remainder virgin guinea-pigs. It will be noted that the length of the oestrous cycle in the last group averaged 15·7 days, as compared with 16·5 days, the average in the other four controls. The average duration of oestrus was also short in the virgin guinea-pigs, 1·6 against 1·73 days. These results are in complete agreement with those of Stockard and Papanicolaou (16), who first used the method in the normal guinea-pig.

In the thyroid-fed animals the average length of the oestrous cycle was 16·1 days, and showed no significant change from the corresponding normals, but there was a definite increase in the duration of the oestrus to an average of 4·7 days. Our observations are somewhat incomplete in regard to the length of time during which the animals were under observation, and our experiments with the mouse, which is a more suitable animal for such experiments. having an oestrous cycle of four to six, instead of fifteen to seventeen days in the guinea-pig, were carried out for a much longer period.

In these later experiments we observed our thyroid-fed animals both before and after thyroid feeding, as well as control animals examined daily over the whole period to exclude any possible seasonal variation during the time of the experiment (240 days).

The results are graphically recorded in figure 5. Since the smears were made at the same time on each successive day, the records give a fairly accurate picture of the occurrence of oestrus. The animals were divided into three groups, each containing five animals. Each group was kept in a separate pot. A and B were the experimental animals and C the controls.

The control animals during the whole period had an average length of cycle of 6·7 days, and an average duration of oestrus of 2·4 days. During the first 72 days, in which no thyroid was administered to the experimental animals, the figures for the controls were 6·3 and 2·2 days. During the second period of 115 days the average figures were 6·8 and 2·8 days, and during the last period of 53 days the averages were 6·9 and 2·3 days. Apart from individual variations these figures do not suggest any marked seasonal change, though the increasing age of the animals may account for a small prolongation of oestrus and lengthening of the whole cycle.

The thyroid-fed animals during the first period when no thyroid was administered, had an average length of oestrous cycle of 6·5 days and an average duration of oestrus of 2·2 days, which did not differ significantly from the averages of the controls, 6·3 and 2·2 days. During the second period of 115 days there was a definite increase in the cycle to an average of 12·9 days, while the oestrus itself was increased to 4·2 days. The change was sudden in its onset, occurring within

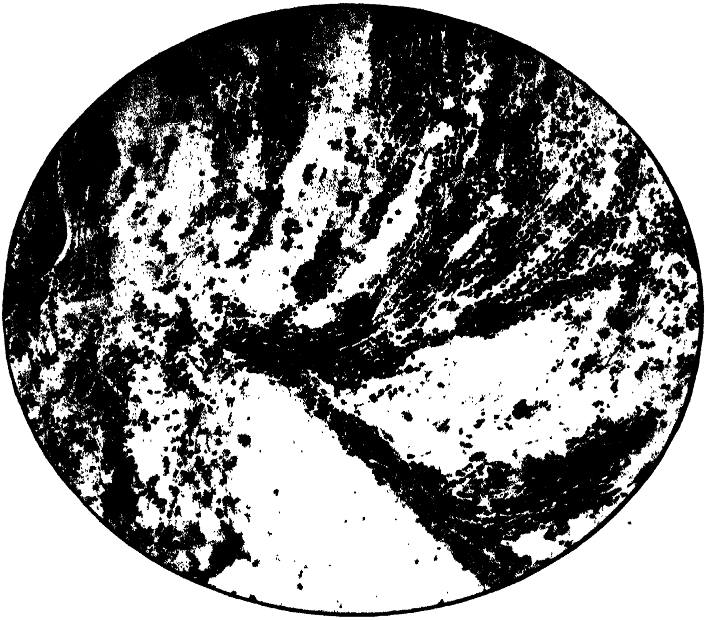

Fig. 3. Magnification 59.

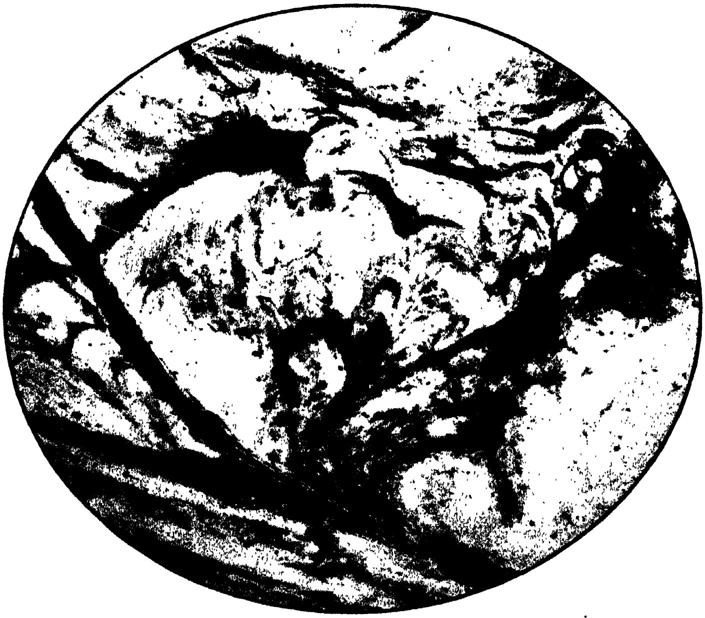

Fig. 4. Magnification 42.

Micro-photographs of typical smears at the various stages of the cycle in the mouse.

the first few days after thyroid feeding had commenced. Individuals showing irregularities in rhythm showed them more markedly when under the influence of thyroid. The first change obvious during the first 40 days was an increase in the length of the oestrus without any striking increase in the dioestrus. After this time the lengthening of the whole cycle was due almost entirely to lengthening of the dioestrus, and the oestrus tended on the whole to be shortened or at all events not notably increased. A comparison of the average figures for the first 40 days with those for the remainder of the period shows this in striking fashion, the length of the oestrous cycle and the duration of oestrus during the former being 9·4 and 4·3 days respectively, and 16·4 and 4·0 days respectively for the latter.

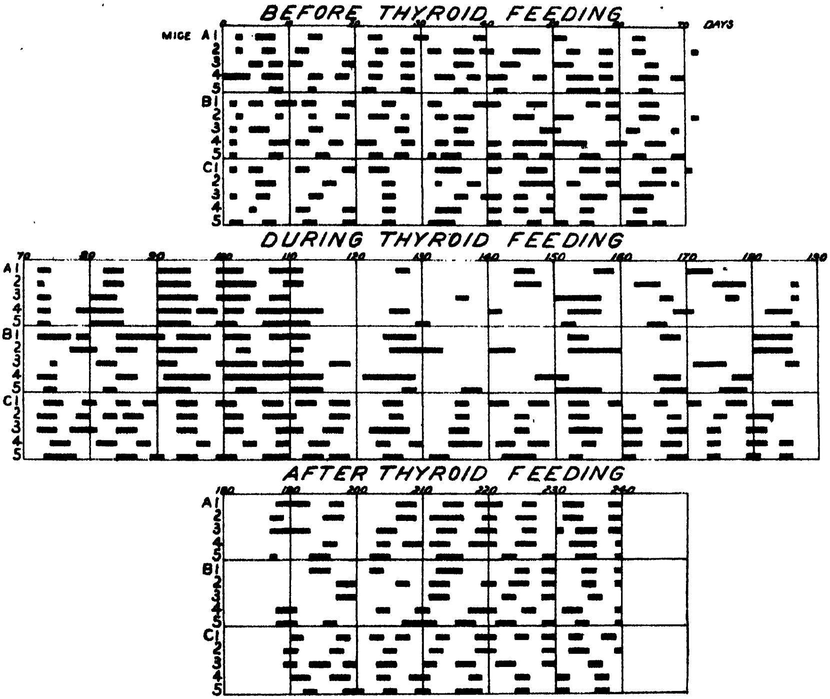

Fig. 5.

With the cessation of thyroid feeding there was a rapid return to normal rhythm. For the whole period of 53 days the average length of the cycle was 7·8 and the duration of oestrus 2·9 days, as compared with corresponding figures for the controls, 6·9 and 2·3 days. The return to normal rhythm was complete

23 days after feeding with thyroid was discontinued; the average figures for the last 30 days being 6·7 and 2·4. The average figures for the last 40 days were 7·2 and 2·7 days, so that after 13 days the reversion to normal rhythm was almost complete.

It is not possible as yet to advance a definite explanation of these undoubted effects of thyroid feeding, since we know nothing with certainty of the manner in which the cycle is normally controlled. Many observers, following Beard (5) and Fraenkel (8), consider that the corpus luteum is of primary importance in the determination of the characteristic periodic changes in the genital tract. Leo Loeb (13) has brought forward experimental evidence in favour of such a view, though his results have been severely criticized by Stockard and Papanicolaou (16). Most writers, however, admit that a hormone elaborated by the corpus luteum appears necessary for the normal development of the uterine and vaginal mucosae, and that the absence of this secretion leads to regression and degeneration of the uterine tissue. The Graafian follicles have also to be considered in this connection, for, though the corpus luteum undergoes degeneration at the height of oestrus, the follicles at this stage attain their greatest development. Allen, Doisy, and their collaborators (2, 3) claim that they have isolated from this source, though not from the corpus luteum, a hormone which on injection into spayed mice and rats brings about the usual phenomena of oestrus.

It remains an open question whether thyroid extract has a specific effect on the reproductive organs, either directly in the production of ovarian hormones, or indirectly by sensitizing the uterine and vaginal mucus membrane to them; or whether it exerts its effect by its influence on general metabolism or on the activity of other ductless glands which play a subsidiary part in the mechanism of reproduction.

CONCLUSIONS.

1. The administration of thyroid extract causes in mice an increase in the duration of oestrus and in the length of the whole cycle.
2. In the guinea-pig there is a marked change in the length of oestrus but no striking increase in the duration of the cycle.

ACKNOWLEDGMENTS.

We are deeply indebted to Dr. C. H. Kellaway, the Director of the Walter and Eliza Hall Research Institute, for his stimulating interest and help. Our best thanks are due to Miss Cecil Maudsley, of the Institute, who prepared the micro-photographs, and to Mr. E. H. Brownbill, of Queen's College, who spent much time in the preparation of our tables.

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LEGENDS.

Micro-photographs of typical smears at the various stages of the cycle in the mouse.

1. At the height of oestrus.

2. At the fourth stage of oestrus, when the leucocytes predominate and the uterine cells are disappearing.

3. In dioestrus, shortly after oestrus.

4. At a somewhat later period in dioestrus.

Figure 5. Graphical representation of observations on the duration of oestrus in the mouse.

ON THE INADEQUACY OF THE CONCEPTION OF THE NEURONE AS THE UNIT OF CONDUCTION OF THE NERVOUS SYSTEM

by

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I propose in this paper to discuss certain aspects of the physiology of the nervous system, which, while they have previously been upheld by Apáthy and Bethe from purely morphological evidence, have failed to gain acceptance by physiologists; the question, namely, whether a single nerve *fibre* is capable of transmitting several impulses simultaneously. The evidence hitherto adduced in favour of this conception must be regarded as inadequate; in particular, in view of the clearly established "all-or-nothing" principle for the contraction of a muscle fibre when functioning under constant mechanical conditions, no good reason has been shown why a single efferent neurone should be capable of transmitting many impulses simultaneously, rather than one, to a muscle-fibre, when that fibre, as we know, is incapable of responding to the stimulus by graded intensity of contraction.

Our present knowledge of the structure of a motor end-plate, and its relation to the two excitable membranes of the muscle fibres, sheds an unexpected light on this question; and I believe that the relation of the end-plate to the muscle fibre becomes intelligible to us only on the assumption of the existence of numerous conducting paths within each motor neurone. During recent years, moreover, a number of observations have been made on the physiology of the sense organs and of reflexes, which seem explicable only on the view that the single neurone must contain numerous subsidiary conducting channels within it; and in the present paper I shall try to establish the following points:

1. The afferent neurone from a peripheral receptive cell responds by graded activity to graded strength of excitation of the receptor; and this graded activity of the afferent neurone is manifested, not by gradation in the frequency with which impulses pass along it, but by a gradation in the *size* of the disturbances propagated.

2. Since, however, the physical process of conduction is one of "all-or-none" intensity, it will follow that a gradation in the size of the disturbance propagated

must have as its basis the excitation of a graded number of conducting paths within each neurone. This will render it highly probable that the neurofibrils, which can be shown not to be "fixation artefacts," are the actual conducting paths.

3. Increase of reflex muscular response to increase in intensity of receptor stimulation depends upon the excitation of a larger number of efferent motor neurones (cf. Sherrington) (26), in addition, of course, to the summational effects of tetanus. The neurofibrils in the efferent neurones serve possibly as efferent paths for various reflexes which involve the same effectors, but are elicited from different receptors; it seems also as if antagonistic reflexes which employ the same effectors may excite them through different neurofibrils of the motor neurones. This will involve a partial modification of the principle of the Final Common Path.

General Remarks on Neurofibrils.

The discovery of neurofibrils we owe to Max Schultze; the theory that they, rather than the nerve fibres, are the actual conducting elements of the nervous system, originated in the work, also of Schultze, but especially of Apáthy (3) and of Bethe (6), who by inventing special staining methods have been able to demonstrate that the neurofibrils form continuous, unbroken paths within the neurones, showing, apparently, no anastomosis with one another, and having the essential property, therefore, of independent conducting elements. Bethe has sought to demonstrate that they are the only *continuous* structures within the fibres, the perifibrillar substance being discontinuous at the nodes of Ranvier; this would be strong, though not conclusive, evidence that the conduction takes place within the fibrils, but it cannot be said that his demonstration has been satisfactory. When one observes, as Bethe has done, that nerves which have been fixed in osmic acid, and especially fibres which have previously been stretched, are devoid of perifibrillar fluid at the nodes, it is clearly impossible for us to conclude whether this represents the real conditions within the living fibre, or whether it is a post mortem shrinkage effect. We cannot even positively assert that a continuous neuroplasm is essential for conduction; on the contrary, the conception of the accumulation of ions on semi-permeable membranes plays a large part in present-day theories of protoplasmic excitation. The second line of evidence introduced by Bethe, though strongly suggestive, is still inconclusive. It rests upon his observation that if a small region of a nerve fibre be compressed, then the capacity of the fibre to conduct impulses ceases at the moment when the neurofibrils lose their primary staining reaction (primäre Färbbarkeit). But if we assume, as many do, that the process of conduction involves an interaction between a membrane (fibril) and its surrounding fluid, then clearly, cessation of conduction on disorganization of the neurofibrils can throw but little light upon the question, whether a single impulse of unalterable intensity is travelling through the neurone in the perifibrillar fluid, or whether an impulse is travelling in one or more of the fibrillae, thus permitting of a gradation in the intensity of the activity of the neurone, though not, of course, of its individual fibrils. The further observation of Bethe (p. 261), that a nerve fibre can still conduct with undiminished strength, even when compression has caused an almost total obliteration of perifibrillar substance from the region of compression, has little meaning at the present day in view of the discovery by Adrian (1) that a propagated disturbance, after undergoing almost total obliteration in a region of decrement, may immediately regain its maximal intensity on re-entering normal nerve. We must conclude, therefore, that while the evidence produced by Bethe is very suggestive, it has failed to demonstrate that the individual neurofibril, rather than the whole neurone, is the elementary conducting unit of the nervous system.

These observations of Apáthy and Bethe have encountered the most serious charge that can be laid against a histological observation—that of artefact. Basing their conclusions on the ultramicroscopic examination of isolated living nerve cells, bathed in serum or cerebro-spinal fluid, Mott (22) and Marinesco (20) conclude that the neurofibrils do not exist as such in the nerve cells during life, but that they are products of the coagulation of the cell cytoplasm, formed subsequent to the death of the cell. This view is accepted in Bayliss' "Principles of General Physiology." It seems to me, however, that this conclusion is entirely unjustified; microscopic examination of living cells can reveal the presence of structures within the cell-body only when the refractivity of these differs markedly from that of the remainder of the protoplasm. Anyone who has ever experienced how difficult or impossible it may be to detect so characteristic a structure as the nucleus in certain living individuals of amoeba, will attach but little weight to negative observations made upon living material. It may be noted, indeed, that in many of Marinesco's microphotographs nuclei are either indistinct or quite invisible. On the contrary, it is pointed out by H. Freundlich (12) that while the particles of certain colloids, such as gold, provided they are large enough, are readily visible under the ultramicroscope, "on the other hand, the particles of colloidal protein or starch solutions are generally invisible, since the solution molecules here contain so much water that their optical properties closely resemble those of the surrounding fluid. The ultramiscroscope is therefore a powerful aid in the recognition of the colloidal nature of a solution, but we must not infer the absence of colloidal particles from a failure of the ultramicroscope to reveal them." We possess, on the other hand, good evidence that the neurofibrils are actual constituents of the cells. When structures, having an identical appearance, can be revealed by treatment of nerve cells by such diverse methods as fixation with strong formalin, or with dilute osmic acid, on the one hand, or by immersion in exceedingly dilute methylene-blue, in which no real fixation can be said to occur, on the other, then we have very strong evidence that we are not dealing with artificial effects, but that the treatment is revealing structures that are present in the living cell. Further evidence in favour of this view will be given towards the end of this paper.

Others, notably Verworn and Goldschmidt, regard the neurofibrillae as skeletal elements, whose function it is to form a support for the fluid "neuroplasm" in which they lie. It is difficult to obtain any real evidence for or against this view. When we observe, as we can do so clearly in the nerve-nets of small blood-vessels, that a single neurofibril may break out from a nerve fibre and run its own course, it is impossible to state from mere inspection whether the nervous impulses travel in the neurofibril, or whether they are conducted by a minute amount of "neuroplasm" which may surround the fibril. Lenhossek (15) has justly dissented from this view of their function, on the ground that if they were merely skeletal elements, one would scarcely expect such an extraordinary array of fibrils within the nerve cells. It is indeed difficult to see in what way the neurofibrils can form supports for the neuroplasm of the nerve axons, when they lie within that neuroplasm, and run in a direction which is least suited to counteract any pressure that may be exerted upon the nerve. Their disposition within the fibres is such that if they were skeletal elements they should counteract tension; compare, for example, the fibrils sometimes seen in connective tissue. It is obvious, however, that such a rôle, if efficiently carried out, would prove disastrous to the nerve. Moreover, many nerves, such as the internuncial fibres within the brain, are never exposed to tension, yet they are exceedingly well supplied with neurofibrils. We must surely conclude that if, as various authors maintain, neurofibrils were skeletal elements, then they should be situated outside the neuroplasm, and not within it. If their function were to maintain an organization of the fluid or semifluid contents of the axon, one might expect to find a radial distribution of neurofibrils; but it would be difficult to see in what manner a *single* neurofibril, traversing a very bulky perifibrillar fluid, such as we find in the motor neurones of *Hirudo*—see Bethe's book (6), figs. 2 and 3—could in any way act as a support for the perifibrillar fluid.

Lenhossek (16) likewise attributes a skeletal supporting function to them, but believes that they exert this supporting function only during embryonic life. As the neuroblasts grow out from the embryonic nerve cord, they meet strong resistance from the tissue through which they grow; he believes that the neurofibrils develop, in order to impart a rigidity to the neuroblasts sufficient to enable them to overcome this resistance. Their persistence in the adult animal he explains largely on the ground that they may be required for regeneration purposes by the nerve, in case of injury. It is clear, however, that the theory of Lenhossek will not account for the occurence of neurofibrils within nerve cells which are devoid of long axons, such as the second type of Golgi cells.

While, then, each of these theories has something in favour of it, it seems that the evidence adduced in support of any one of them is inadequate; it is, indeed, almost impossible to form a conclusion on this point by mere microscopic examination of the fibrils. Nevertheless, I believe that there are a number of well established physiological observations which are quite incompatible with the view that the neurone is incapable of grading the intensity of its response. If this point can be proved, then, in view of the fact established by Verworn and his school, and by Adrian, that the physical process of conduction is, in normal nerve, one of "all-or-none" intensity, it will follow that underlying this gradation there must occur the excitation of a graded number of conducting paths within the individual neurones. If this can be proved, it will constitute very strong evidence that the neurofibrils are indeed the elementary conducting units, as Apáthy and Bethe have maintained.

It must be admitted, at the same time, that in ascribing a conducting function to the neurofibrils, a number of difficulties are encountered. In the well-known figures of ganglion cells prepared by the reduced silver method, and depicted by Cajal and others, the fibrils are usually not shown as isolated from one another, but, on the contrary, appear entangled with one another by a very delicate network which pervades the cell. But it is almost certain (4, 13) that we are dealing here with a fixation artefact, the various fibrils adhering together as the cell cytoplasm coagulates under the influence of the fixative. Bethe, on the contrary, is able to make preparations in which no such anastomosis occurs. This point is especially strongly insisted upon by Jäderholm (13), and by F. M. Ballantyne (4), who believe they can trace individual neurofibrils right through the ganglion cells, without their undergoing any extensive anastomosis with adjacent fibrils. I believe that this conclusion is only partly correct, and shall discuss it more fully at the end of this paper.

The same objections may be raised against observations in which anastomosis of neurofibrillae is described within the nerve fibre. Bethe, on the other hand, has observed them running entirely independent paths within the fibres. I believe I can confirm this observation, although it must be admitted that it is usually an almost impossible question to decide with certainty, so closely do they run together.

Histologists are almost unanimous in their opinion that in the terminal end-plates of motor neurones a true anastomosing network of neurofibrils occurs. The same criticism applies here also; moreover, Boeke (8) has been able to obtain preparations in which the fibrils proceed independently within the end-plate, and terminate each in a small loop; and he inclines to the view that no real anastomosis of fibrils occurs in this situation.

I have examined the neurofibrillae in the motor nerve endings, in the frog, and in contradiction of the work of Cajal, can entirely confirm Boeke's conclusion. By means of Bielchowsky preparations, I find that the neurofibrils, separating out from one another in the terminal end-plate, run an entirely independent course, and end in little loops. The "loops" seem to be identical with the end-plate "knobs" referred to below. Two such nerve endings, taken from the frog's biceps muscle, are illustrated in figure 5. When we remember the great opportunity presented to the neurofibrillae for sticking together during the coagulation of the fluid cytoplasm in which they lie, then we are justified, I believe, in laying greater stress upon preparations in which the neurofibrils run an independent course within the neurone.

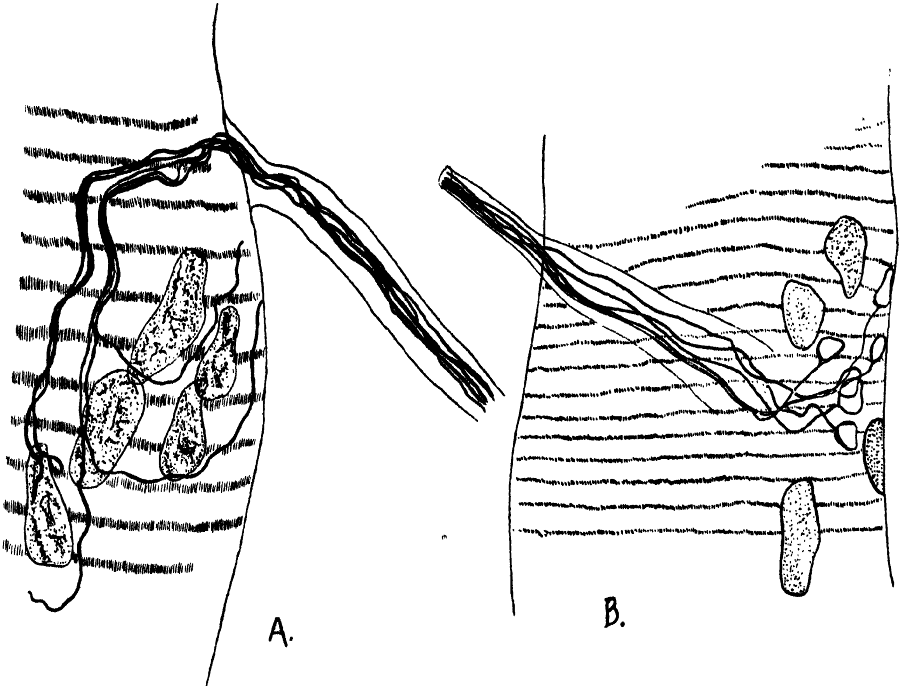

Figure 5. Distribution of neurofibrils in motor end-plate; from biceps muscle of frog (*Hyla aurea*).

A. Note that the neurofibrils show no tendency to form anastomosing networks. In this preparation only one end-knob has been revealed.

B. This specimen, like the last, is from a longitudinal section along the muscle fibre, and only part of the end-plate is seen in the section. Note, again, that the neurofibrils run a perfectly independent course within the end-plate, and also within the axon. Note also the neurofibrils ending in small loops ("end-knobs").

(Bielchowsky reduced silver method.)

A much more real difficulty lies in the fact that many neurofibrils within the ganglion cells do not appear ever to enter the axon, but travel, on the contrary, from one dendrite to another. This would be a very serious objection. I do not believe, however, that the observation is as well established as the figures of Bethe and of Jäderholm may lead us to expect, and shall discuss this more fully below.

On Gradation in the Intensity of Sensory Excitation.

The vertebrate spinal cord is a structure of such extreme complexity that it is well nigh impossible from a study of its reactions to decide whether we must consider the neurone as the unit of conduction, or whether we must attribute a capacity for conduction to its constituent neurofibrils; when, for example, we observe extensive gradation of reflex response to a gradation of intensity of stimulation of a peripheral skin-point, we are not able to say definitely whether the afferent neurones have conducted disturbances of graded intensities, or whether central summation and facilitation alone can account for it. For this reason we are likely to derive much more definite results from a consideration of the behaviour of an organ of whose structure and mechanism we have a more precise knowledge. I believe that the human ear lends itself well to this purpose.

The cochlea may be considered as a strip of sensory epithelium, the excitation of any given hair-cell, or set of hair-cells, producing an auditory sensation of a particular definite pitch. The very important histological fact has been discovered by Cajal (9), by Kolmer (14), and by Bielchowsky and Brühl (7), that each hair-cell is innervated by a *single* nerve fibre, which is continuous with a single ganglion cell in the spiral ganglion. The nerve fibres form at the base of the hair-cell each a cup-like expansion, which can now be seen to be composed of numerous very delicate neurofibrils, which closely embrace it (Kolmer).

Of the two theories of hearing that have prevailed in the past, that of a peripheral and that of a central analysis of the sound, the former alone can be considered at the present day; the latter theory, especially in the form in which it was propounded by W. Rutherford (1886), is definitely contradicted by the fact that nerve fibres are unable to transmit impulses at the high frequency that it demands. Moreover, as has recently been shown by Wilkinson and Gray (32), contrary to the statement of Keith, the cochlea is a structure which is capable of responding to sound waves of different frequency and intensity, very much in the manner postulated in Helmholtz's theory of peripheral resonators. Assuming, then, the correctness of the theory that the analysis occurs peripherally, we can further state that the excitation of a certain receptive cell, or group of such cells, produces always a sensation of a note of particular pitch. The clearest evidence for this statement lies in the phenomenon of auditory fatigue, where the ear becomes fatigued to a sound of particular pitch to which it has been subjected, but remains unaffected for all other notes. Irrespective, then, of whether we adopt the resonance theory of Helmholtz, or the much less probable sound-pattern theory of Ewald, we can conclude that sound waves of a particular frequency and amplitude excite in us a sensation of a note of particular pitch and loudness, by stimulating with a certain intensity a certain localized hair-cell, or group of hair-cells, in the cochlea. This is the view held by most physiologists; I have here merely repeated the facts on which it is based, in order to be more certain of the point which underlies the subsequent argument.

It has long been an obscure problem in the physiology of hearing how the central auditory mechanism can be apprised of the different degrees of loudness of sounds, when the auditory nerves are unable to conduct from the ear impulses of graded intensity. An ingenious explanation, based on the fact of the reduced excitability of nerves during the relatively refractory period, has been offered recently by Forbes and Gregg (10). I do not believe, however, for reasons that will be given below, that this explanation is an adequate one.

Underlying a gradation in intensity of sensory response, we may imagine three different processes: (1) There may occur an excitation of a varying number of afferent neurones; or (2) the impulses, whether of normal or of sub-normal intensity, may pass along the nerve at graded frequency; or (3) the disturbance propagated within the single neurone may be of graded intensity.

1. Gray (32) has considered the first of these processes as playing a large part in the mechanism of the recognition of degrees of loudness. Taking into consideration the fact that, in tactile sensations, with increasing pressure the increase is judged as occurring only at the actual point of application of the object touched, although receptors over a considerable surrounding area must be excited (as seems to be proved by the blanching of the skin capillaries here), he points out that a similar phenomenon must occur within the ear, for since the vibrating fibres of the basilar membrane are loosely bound together, vibration of the fibre, which is in perfect resonance, will be accompanied by a partial spread of the vibration, and the extent of spread will be the greater, the greater the amplitude of swing of the maximally stimulated fibre. He suggests that a gradation in the number of fibres thus secondarily brought into action may serve as the basis for discrimination of intensity of auditory excitation. There are, however, several fatal objections to this view. In the first place the well-established fact of auditory fatigue proves beyond doubt that in hearing a very loud note, some *single* irritable cell, or group of cells, has been functioning with great activity; we must conclude that the basis of graded intensity of hearing is a graded activity of a single sensory unit, and not a gradation in the number of sensory units stimulated. A further serious objection lies in the fact that a large number of soft notes nearly but not quite in unison, should give rise to a sensation of a note of maximal intensity; we know, however, that they merely produce an unpleasant discord. A third objection is that the theory will obviously not apply to the eye. As a modification of this theory it might be

urged that the number of upper harmonics that attain threshold value might serve for the discrimination of loudness; it is well known, however, that they will affect merely the quality of the note. From these considerations, then, we must conclude that *gradation of loudness of hearing does not depend upon a variation in the number of auditory nerve fibres excited.*

2. A more obvious suggestion, in that it would permit of central summation effects, is that gradation in the *frequency of passage* of impulses along the auditory nerves, whether of normal or of subnormal intensity, might underlie gradation in the intensity of the sensation experienced.

Let us consider first the case of gradation of frequency of impulses of *normal* intensity. Suppose a pure note is sounded repeatedly at very short intervals and at low intensity; as we sound the note more and more rapidly it will eventually be heard as a continuous note, whose loudness we can increase by striking it with greater force. There are two reasons why the note may become a continuous one: it may be that the receptive cell is transmitting to the auditory centre the maximum number of impulses that the auditory nerves can carry, in which case increase of the loudness of the note cannot be due to any further increase in the frequency of passage of impulses of *normal* intensity along the nerve. On the other hand, we may assume that the note becomes continuous long before the auditory nerves are transmitting normal impulses at maximum frequency. Suppose that in this case we now sound the note twice as rapidly as is required to produce the sensation of a continuous note; the note should now sound twice as loud, and in the limit, any note which is sounded as a continuous, sustained note must be one of maximal intensity, which we know to be untrue. We must conclude that gradation in the intensity of hearing cannot have as its basis gradation of frequency of passage of *normal* impulses along the fibres of the auditory nerve.

A very ingenious suggestion has, however, recently been put forward by Forbes and Gregg (10) to explain gradation in the strength of sensory excitation. From the researches of Keith Lucas and E. D. Adrian (2, 18) we know that by using stimuli of intensity above normal threshold value, nerves can be forced to conduct impulses during the subnormal conduction phase; and by increasing the intensity of the rapidly repeated stimuli, larger and larger numbers of impulses, each of subnormal intensity, can be excited in the nerve per unit of time. Forbes and Gregg suggest that the receptive cell may act in the same manner as our artificial stimuli, and that the stronger the excitation of the receptor the larger the number of impulses that are imparted to the sensory nerves in a unit of time. They conclude that "if the sensory receptors provide to the afferent fibres sustained stimuli of graded intensity, the brain may be apprised of the intensity of the peripheral stimulus by the frequency with which the impulses come to the centre over the afferent nerve fibres."

Against this suggestion must be raised the objection, firstly, that if any increase in the number of impulses thus reaching the auditory centres occurs, the individual impulses will be of subnormal intensity, and it is doubtful if any beneficial results will accrue by such impulses reaching the auditory centre; it is well known, for example, that if a strong stimulus be applied to the heart, while it is in the relatively refractory condition, the contraction that is elicited is of correspondingly diminished size. Furthermore, if we assume with Keith Lucas that the "synapses" represent regions of decrement, then the increased frequence of passage of subnormal impulses would lead to a condition of inhibition (Wedensky inhibition), rather than of increased central excitation.

Much more conclusive evidence against the suggestion of Forbes and Gregg is to be found in some remarkable observations by Lord Rayleigh (23), and by More and Fry (21), on the capacity of the human ear to recognize phase difference. In these experiments it was found that if two sounds of equal pitch and intensity, but having a difference in phase, were led, the one to one ear, the other to the other, then the sound was invariably judged as coming from that side which had the lead-in phase. If, as in the experiments of Rayleigh, the sound emitted by two tuning forks, tuned almost but not quite to the same pitch (the beat occurred once in about five to fifty seconds), were led off independently to the two ears, then when the lead of phase was on the right side, the sound was judged with unerring precision as coming from the right side; as the lead in phase gradually changed to the left side, the direction of the sound seemed as surely now to change with it. This phenomenon could only be observed with frequencies below about 700 d.v. per second.

In order to account for this extraordinary fact, Rayleigh was led to accept the "telephone" theory of Rutherford in preference to the resonance theory. Nevertheless, our present-day knowledge of the properties of a nerve fibre prevents us from adopting this conclusion. The real explanation doubtless lies on the lines already implied in Rayleigh's conclusion. When the lead of phase is on the right side, the condensation will attain threshold value on the right side slightly earlier than on the left; and we must assume that excitation of the homorhythmic hair-cell, and therefore of the corresponding auditory neurone, will occur slightly earlier on the right side than on the left. This conclusion is in no way invalidated by the fact that it requires considerably more than one vibration to excite the sense cells. Hence impulses will reach the auditory centre from the right side slightly in advance of those from the left, and this seems to be the basis on which judgment of direction is formed. But this carries with it the very important implication that *the frequency with which impulses pass along the auditory neurone from the receptive cell of the one ear, is equal to that with which they pass along the auditory neurone from the corresponding receptive cell of the other ear, provided the stimuli have been of equal intensity.* It might seem at first sight incredible that a living tissue should behave with such astonishing precision; nevertheless, the physical observation proves that such a thing does occur.

Suppose, now, a source of sound to be so situated, that the intensity of excitation of the two ears is unequal; and on the assumption that there will now occur a variation in the frequency of passage of impulses along the corresponding auditory neurones of the two sides, let us suppose that the source is so situated that the right ear (nearer) transmits 550 impulses per second to the central auditory mechanism, the left only 500. It is clear that at the end of every one-hundredth part of a second the lead of impulses reaching the auditory centre will change from one side to the other, consequently the sound would seem to come momentarily from one side, momentarily from the other, and no localization of the sound would occur. We know with certainty that this is not the case, and must therefore further conclude that *the frequency with which the impulses pass along the auditory nerves is independent of the intensity of the excitation.*

3. From these considerations, then, I believe we are justified in stating that neither the excitation of a graded number of afferent neurones, nor a gradation in the frequency of passage of impulses along the neurones, can serve as the basis for a discrimination of intensity of peripheral excitation; but that, on the contrary, it is the *size* of the disturbance propagated in each neurone that underlies this phenomenon. Nevertheless, we know that the actual process of conduction, if it occurs at all, is for normal nerve one of unalterable intensity; underlying a gradation in the size of the disturbance propagated in a single neurone there must occur, therefore, a gradation in the *number* of elementary conducting units excited within the neurone, and it does not seem unjustifiable to assume that these units are the neurofibrils. We reach our final conclusion, therefore, that *the number of neurofibrillae excited within a single auditory neurone serves as the basis of our judgment of the intensity of peripheral auditory excitation.* This may possibly throw some light upon the fact that a continuous increase in the intensity of peripheral excitation is accompanied by a discontinuous increase in the sensation (Weber's law).

The Physiology of Motor End-plates.

The end-plates by which motor neurones terminate upon skeletal muscle fibres reveal a number of features which appear to be incompatible with certain well-established principles of stimulation of excitable tissues, unless there exist numerous conducting paths within these end-plates. The long, branching end-plates of frog and snake muscle are especially instructive.

When we examine good preparations of these end-plates we often find that the axon of the nerve, immediately before entering the muscle fibre, divides into two or more parts, which then terminate separately upon the fibre. In figures 1 and 2, for example, are shown two end-plates, which have broken up into four parts just before penetrating the sarcolemma. The nerve endings in the sartorius muscle of the frog are usually formed by bifurcation of the axon, the two parts then undergoing extensive branching on the fibre. In figure 3 is shown an end-plate, taken also from the frog's sartorius, which consists of three main branches. If we assume that the axon as a whole is the conducting unit, then it follows that the muscle fibre on excitation receives as many stimuli simultaneously as the motor nerve has terminal branches. But it seems to me that the histological observations allow us to go further than this.

In a previous paper (30) I have figured the nuclei[1] of the motor end-plate as connected directly with the membranes of Krause. These end-plate "nuclei" were also observed to be connected with very delicate fibrils, which could be traced for a short distance backwards into the terminal expansion of the motor nerve. The figure, which I have redrawn here (figure 2), shows a very important feature, whose meaning I did not at the time appreciate, namely, that many of the "nuclei" of the end-plate are connected, not with one, but with both the excitable membranes. The fact, firstly, that very delicate fibrils (which are probably the neurofibrils) can be observed to communicate with the end-plate "nuclei"; and, secondly, that these end-plate "nuclei" are connected, apparently, with both excitable membranes, seems to suggest that the excitation of a single neurofibril is sufficient to stimulate the two excitable membranes, and therefore to produce a single muscle twitch. In figure 1 is shown a motor end-plate from the mylohyoid muscle of the frog, in which the connection of the end-plate nuclei with very delicate fibrils (presumably neurofibrils) from the motor neurone, is seen. Concerning the connection of the end-plate "nuclei" with *both* excitable membranes, it must be admitted that this cannot always be observed; and, indeed, a considerable number of the "nuclei" shown in figure 2 are connected apparently only with one membrane. But we must remember that the methods at present available for demonstrating nerve endings are, from the cytological standpoint, crude, and possibly the connection of these nuclei with one membrane only may be due to shrinkage. It seems to me, therefore, that we have considerable evidence to show that the neurofibrils of the motor neurones part company terminally, and communicate each with an "end-knob," which then communicates often, perhaps always, with the *two* excitable membranes of the muscle fibre, as shown diagramatically in figure 4.

But whether we are willing to accept these finer histological details, with the implication that there are a large number of separate conducting paths to each muscle fibre, or whether we merely conclude that there are a small number of such terminal paths, according to the number of parts into which the motor neurone breaks up terminally, in either case we see that if the neurone is the unit of conduction of the nervous system, then the single muscle fibre will receive several stimuli simultaneously. Especially instructive figures will be found in

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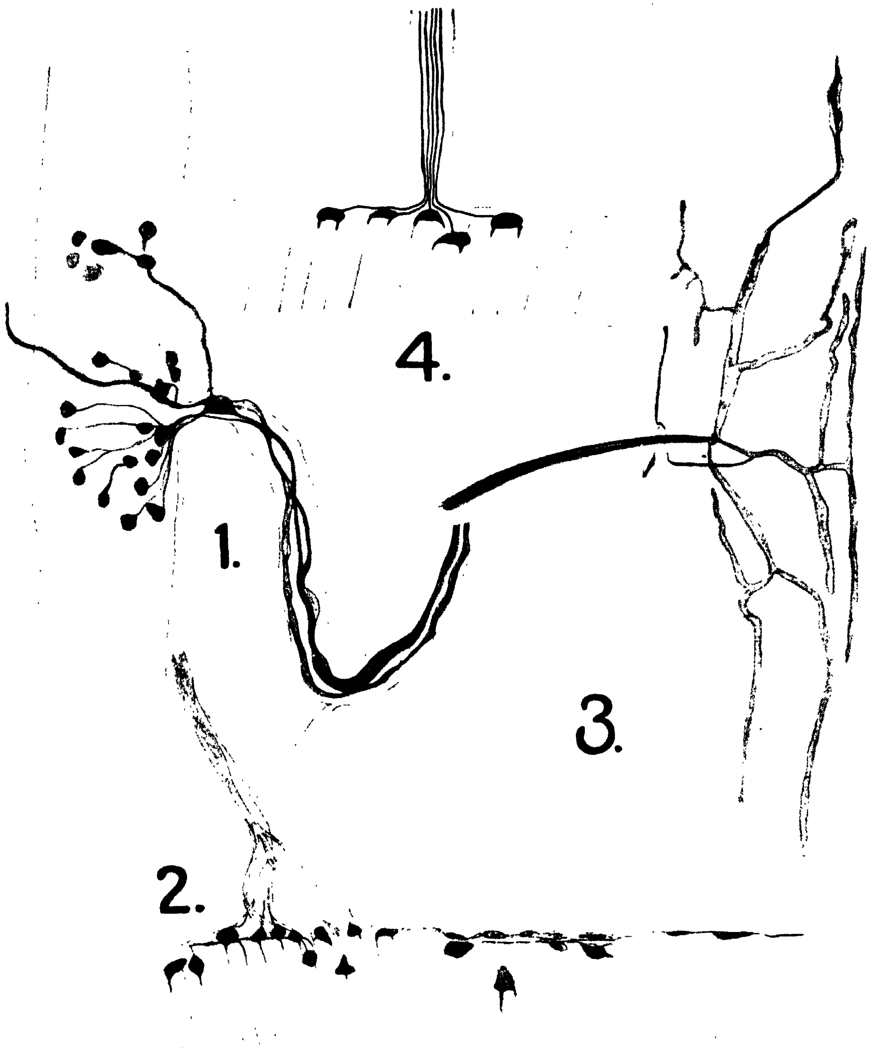
[1] The term which I have previously (30) used for these structures is misleading; they are quite different from the "sole-nuclei" of the end-plate, in that they are much smaller, and do not appear to contain chromatin. The term "end-knob" would be more suitable.



1. Motor nerve endings on muscle fibre (from mylohyoid of frog). Note the "end knobs" connected by fine fibrils with the axon.

(Gold chloride preparation.)

2. The same, in side view. Most of the end knobs are in direct connection with the excitable membranes; at least five of the end-knobs are in connection with both the excitable membranes.

(Gold chloride preparation.)

3. Motor nerve ending on fibre of frog's sartorius.

(Gold chloride preparation.)

4. Diagram to show the neurofibrlilar organization of a motor end-plate.

a paper by Sihler (24), who points out that in frogs and snakes the end-plate "sole" protoplasm, which is so characteristic of mammalian motor endings, is absent, and that excitation of the muscle fibre takes place through *numerous points of contact* between the nerve ending and the muscle fibre. What is the meaning of this curious fact?

It might be urged that the stimuli transmitted to the muscle fibre by the individual branches of the motor neurone are not strong enough to excite individually, but that several stimuli, each of subliminal intensity, reinforce one another, producing a stimulus which is now of adequate strength. But the researches of Keith Lucas and Adrian (17, 18) have shown that such a summation of inadequate stimuli can occur only if these stimuli are applied at the same point. Such a summation is believed to be a strictly localized, molecular effect, due, it is believed, to an accumulation of ions on the surface of a membrane, and the distance apart of even the finest branches of the motor end-plate far exceeds the range of action of molecular forces. If, on the other hand, we assume that the stimuli transmitted through the terminal branches of the motor neurone are above threshold value, then the end-plate is equally difficult to understand. The excitation wave from a stimulated point, situated anywhere along the fibre, spreads in both directions, from which we must conclude that one branch of the end-plate is sufficient to excite the muscle fibre, and all the others are superfluous. If there are more than two such terminal branches, then it can be shown that if the neurone is the unit of conduction, then all these branches, except the two outermost, can never play any part in the excitation of the muscle fibre, for the excitation to which they give rise will enter tissue left refractory by the passage of impulses from the two outermost branches, and so will be obliterated.

From these considerations we see, then, that a branching motor end-plate, or indeed even a continuous but spreading motor end-plate, applied to a series of successive points along a double helicoidal membrane (30), is a structure which in the light of our present knowledge of excitation is meaningless, unless there occur within it separate conducting channels, each independently excitable from the central nervous system. The histological observations are exceedingly difficult to make, for we do not at present possess an entirely satisfactory technique for revealing the finer details of motor end-plate organization; but so far as these observations go, they seem to show that the neurofibrils of the motor nerve are provided each with an "end-knob," and that these "end-knobs" are connected each with the two excitable membranes, so that excitation of a single efferent neurofibril will suffice to stimulate the entire muscle fibre.

From the above theoretical discussion it would appear that the motor neurone comprises not one, but numerous efferent paths to the muscle fibre, and that one only of these efferent paths is employed in a given reflex. A method is available for testing this deduction experimentally. If a motor neurone contains many efferent paths, one only of which is employed in a given reflex, then the current of action for a motor nerve excited reflexly ought to be much smaller than for one stimulated directly with a maximum shock. It seems, indeed, that the theory of multiple efferent paths must stand or fall by this test. Unfortunately, most reflexes do not lend themselves for this experiment: firstly the reflex muscular response is usually very different from a single muscle twitch; secondly, the nerve trunk usually contains numerous motor fibres to other muscles which may not be involved in the reflex; thirdly, the nerve trunk contains both afferent and efferent nerve fibres, so that direct excitation of the nerve may excite many fibres which are not employed in the reflex. The flexion reflex in the hind limb of the cat to some extent avoids these difficulties; it may be elicited by application of a single shock to the afferent (popliteal) nerve. The reflex thus elicited in the decerebrate cat is nearly but not quite as high as the twitch obtained by direct stimulation of the motor nerve (Sherrington, 29). But it is considerably more prolonged, being a brief tetanus rather than a simple twitch. Furthermore, considerably less than half of the fibres in its nerve (peroneal) are afferent (Sherrington, 25).

Data concerning the question before us are available from the work of Forbes and Gregg (10), who have compared, in the decerebrate cat, the monophasic action current in the peroneal nerve, when excited directly and indirectly by maximal induction shocks. They find that the action current of the directly stimulated nerve is very much greater than that of the nerve when excited reflexly. The difference is in part to be accounted for as due to excitation, on direct stimulation, of the afferent neurones. On the other hand, the reflex contraction is not a simple twitch, but a tetanus of very brief duration, so that there will be a pronounced additive effect of the individual action currents, and this will not be diminished by the fact that the impulses in the whole nerve present the character of an overlapping series. The very great amount by which the reflex action current thus observed will exceed that due to a single reflexly elicited twitch (if such were obtainable) may be estimated by comparing the simple and the compound curves given by Forbes and Gregg in the latter part of their paper for summation in the current of action in muscle. For a discussion of the effect of *duration* of excitation, on the size of the galvanometer deflection, see J. F. Fulton (11). These quantitative measurements seem to show, then, that even if we allow for the excitation of the afferent peroneal neurones, *the directly excited action current is very much larger than the reflexly excited.*

Application to Spinal Reflexes.

When we attempt to apply these conclusions to the elucidation of the action of the central nervous system, serious difficulties are encountered. We are dealing here with a structure of such extraordinary complexity, that it is generally quite impossible to form a definite conclusion as to where a particular process is taking place. When, for example, a facilitation or an inhibition has occurred, or when a prolonged reflex becomes fatigued, it is usually not possible to say with certainty to which part of a reflex arc such a process must be ascribed. Nevertheless, certain facts are available on which it seems a general conclusion may be based.

In contradiction to the earlier statements of Wundt, Baglioni, and others, it has been found by Sherrington (27, 28), that many reflexes do not behave in an "all-or-none" manner, but that a very marked gradation in the size of the reflex response, with gradation in the intensity of the excitation may occur. Since this effect may be obtained by stimulation of *minute "skin-points,"* excitation of more or fewer afferent neurones seems to be excluded. If the conclusion reached above, concerning the gradation in the activity of auditory neurones, be applied to afferent neurones in general, then it seems that we may gain considerable insight into the nature of this gradation of reflex response.

When an afferent neurone enters the spinal cord it does not usually terminate upon a single nerve cell there, but on the contrary may continue a considerable distance up or down the cord, giving off collaterals to a very large number of nerve cells. According to independent observations by Bethe (6, p. 55) and Lenhossek (15), *single neurofibrillae separate out from the main bundle of neurofibrillae in the axon of the afferent fibre, and one neurofibril passes into each collateral.* Consequently, with gradation in the intensity of excitation, since there will occur, as we have seen, a gradation in the number of neurofibrillae excited in a single sensory neurone, therefore there will occur a corresponding gradation in the number of efferent fibres stimulated, with a corresponding gradation in the intensity of the muscular response. At the same time, we must not underestimate the effects of central facilitation and summation, in cases in which we are dealing with stimuli of longer duration than a single shock; it seems, nevertheless, that the breaking down of graded synaptic resistances will not entirely account for the gradation. For, if we assume, on the basis of our experience with the ear, that the sensory nerves do not respond by increased frequence of passage of impulses, to increased peripheral excitation, then it will be clear that central summation will not explain a gradation in the size of the *initial* contraction of the reflex response, although a subsequent increase in the response with continued application of the excitation will doubtless have central summation as its basis. Nevertheless, when extensive irradiation of impulses, in response to very intense excitation, occurs, it would be absurd to suggest that the effect would be due merely to excitation of the longest neurofibrillae.

From these considerations, then, we conclude that in response to an increase in strength of excitation of a receptor, more neurofibrils within a single afferent neurone are stimulated. These neurofibrils, separating out from the main trunks of the neurones into the collaterals, within the spinal cord, bring about excitation of more efferent neurones, with a resulting increase in the reflex response. There is one objection which may be raised against this conclusion: if greater activity of the receptor brings about an excitation of more neurofibrillae within the neurone, may it not chance that some of the neurofibrillae, excited even by light stimulation, be those that pass to the more distant muscle groups? It is necessary to assume that such a thing does not occur, and that the longer neurofibrillae, perhaps on account of a greater excitation threshold, are excited only by the strong stimuli. The difficulty is encountered even when we regard the neurone as the unit of conduction, for in this case it would be necessary to assume a greater synaptic resistance for the more distant central branches of a sensory neurone.

The previous discussion on the motor end-plate has led to the conclusion that each muscle fibre is connected, not by one, but by numerous efferent paths with the central nervous system. This constitutes the greatest difficulty that the neurofibril theory has to encounter: we could readily understand the presence of numerous neurofibrils within sensory neurones, but it is not easy to see why so many efferent channels should exist, when apparently one alone would suffice for each muscle fibre.

Before discussing this point it will be useful to refer to one animal—the leech—in which this apparently primitive condition seems to have been realized. The researches of Apáthy and Bethe have show that in this animal the motor neurones possess each only a single thick neurofibril, while the sensory neurones possess a large number of smaller fibrils. At the periphery the fibrils of the sensory neurones part company, and are distributed to *numerous* skin receptors. When these sensory neurones are traced into the nerve cord the neurofibrillae are found to enter the central fibrillary mass (neuropil), where it is no longer possible to follow them individually. From this "neuropil" bundles of fibrillae again collect, and pass into the ganglion cells, where they form a "basket-work," from which a single thick neurofibril arises. This neurofibril passes outwards then in the motor neurone, which therefore has only a single neurofibril. So far as the connection of the sensory and motor neurones is concerned, it is necessary to assume that the fibrils of the bundle which enters a particular ganglion cell, are not identical with those of the corresponding sensory fibre; for under these circumstances it would be a simple matter to trace such a connection, and the neuropil would present the appearance of regular bundles of fibrils, rather than of an irregular, bizarre "Punktsubstanz." The actual morphological homologies of these structures are uncertain; this will, however, in no way affect the main argument.

The nervous system of the leech appears to illustrate in the most vivid manner the principle of gradation of reflex response, which appears from quite different considerations to prevail in the vertebrate nervous system also. Since the neurofibrils of the sensory neurones communicate peripherally with numerous skin receptors, it is scarcely possible to doubt that a gradation in sensory response must occur. It will be a gradation, not indeed to intensity, but to extensity of excitation. It is clear, however, that for tactile stimuli extensity of excitation is, to some extent, a measure of intensity. The greater the peripheral excitation, therefore, the larger the number of neurofibrils in each afferent neurone excited. In the central neuropil a rearrangement of these fibrils occurs, resulting eventually in the excitation of more efferent neurones, i.e., in an increase in the muscular response.

But in the vertebrate, conditions are much more complicated, and the efferent roots contain numerous neurofibrillae. Perhaps this is to be correlated with the vastly greater variety of muscular movements of which these animals are capable; but we do not appear to possess enough data on which a generalization may as yet be based. Nevertheless, if we assume that not the neurone, but rather its constituent neurofibrillae are the actual conducting units of the nervous system, then a number of anomalies which seem to prevail in the physiology of reflexes, may find an explanation.

For example, it has been observed by Sherrington, that after a scratch reflex of a dog has been fatigued by exciting various points of the receptive field, the flexion reflex, which employs the same flexor muscles, can still be excited with ease. This leads to the conclusion that while most nerve cells are very rapidly fatigued, the final common neurone is relatively unfatiguable. Might not the anterior horn cells owe this characteristic to their possessing many instead of but one efferent conducting paths, the one conducting path being employed in one reflex, the other in another, perhaps antagonistic reflex, but involving the same effector? We should bear in mind the extraordinary observation of Sherrington (28), that a reflex, after being totally fatigued, may even under continued stimulation suddenly recover its normal strength, and repeat this several times. It seems impossible that the fatigue should have occurred at the first "synapse"; could it not have occurred at part of the final neurone, and a new path then become laid out, by summation, to another part of this final neurone? If we believe that relatively little fatigue occurs in the anterior horn cells, we should have to face the fact that histologically the most profound fatigue effects can be detected here (Gustav Mann, 19).

Consider the case of the displacement of a reflex by another reflex that employs the same effector, but to a different purpose, e.g., the displacement of the flexion reflex by the scratch reflex. If the terminal neurone is regarded as divisible, the inhibition of the flexion reflex might be conceived as occurring on part of this neurone, while the remainder of the neurone still remained responsive to stimuli from the afferent neurone which evoked the scratch reflex. But if we conceive the terminal neurone as indivisible, then we should have to place the inhibition at some more central internuncial neurone; and the conditions within the cord are so complex that we cannot positively deny this conclusion. But if the inhibition occurs here, rather than at the last motor neurone, then there would clearly be no mechanism for the inhibition of after-discharge from the terminal motor neurone, and yet Sherrington's figure seems to show that the after-discharge characteristic of the flexion reflex is cut off very short when the scratch reflex replaces it—see Sherrington (28), figure 51. We should then have to conclude, either that no after-discharge occurred on the anterior horn cells, or, as an alternative, that these were indeed divisible. Nevertheless, recent work by Ballif, Fulton, and Liddell, (5) seems to show that an after-discharge from the anterior horn cells can indeed occur, and be inhibited. The experiment consists in the inhibition of the after-discharge of the knee-jerk, a reflex in which, it is usually believed, no internuncial neurone is involved.

But in those cases of inhibition in which we are not faced by the above dilemma, we usually locate the inhibition on the anterior horn cell, and the histological observation of Cajal (9), that a very large proportion of posterior root fibres terminate directly upon the anterior horn cells, lends strong support to this view.

The conditions within the cord are, however, so complex that beyond this it does not seem possible, from a study of its reactions, to obtain any critical evidence for or against the theory of the divisibility of the terminal motor neurone. Since the conclusions based on this evidence are as yet too vague, the following histological observations on the gray matter of the spinal cord supply much more decisive evidence.

Histological Observations on the Structure of the Gray Matter of the Spinal Cord.

The following observations on the gray matter of the spinal cord have been made by the use of the reduced silver method of Cajal, and especially by the Bielchowsky technique for revealing neurofibrils. Unborn or newly born rabbits and guinea-pigs were employed.

When good preparations of the spinal gray matter, especially those stained by the Bielchowsky method, are examined, there are to be observed in this gray matter the following structure: (*a*) nerve "cells," of which the anterior horn cells stain especially brilliantly; (*b*) neuroglia structures; in the preparations which I have employed only the nuclei of the neuroglia tissue have stained (see figure 6), so that no difficulty arises in confusing neuroglia threads with fine nerve fibres; (*c*) large nerve axons, which may be observed in small numbers traversing the gray matter; (*d*) enormous numbers of fine, sometimes extraordinarily fine, fibrils, which arise as collaterals either from the posterior root neurones or from the various descending neurones of the surrounding white matter. These fibrils run often independently, in an irregular manner, through the gray matter, but are more usually seen traversing it in smaller or larger bundles (see figure 6). Sometimes it is possible to trace these fibrils very long distances, but sooner or later they usually run out of the plane of the section.

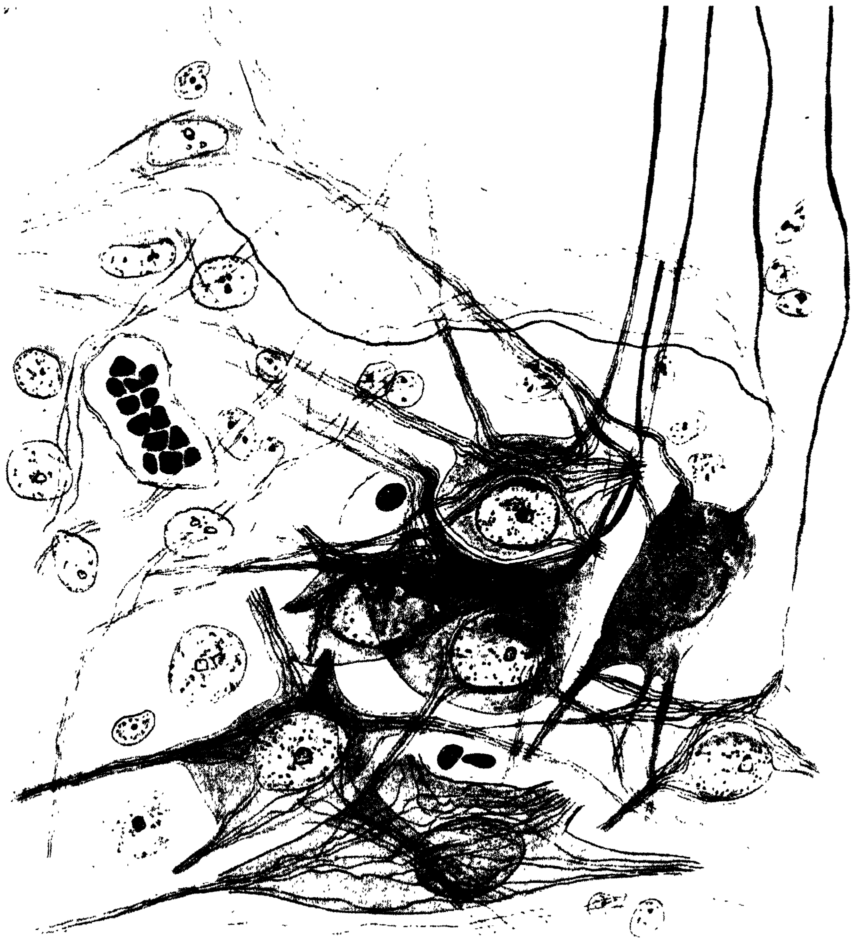


Figure 6. Group of anterior horn cells and surrounding grey matter from spinal cord of rabbit. In the region outside the nerve cells fine fibrils (collaterals) traverse the section, either in bundles or singly; they are frequently cut short as they pass out of the plane of the section. These fibrils can be traced, sometimes for long distances, into the dendrites of the nerve cells; details of the anastomosing system in the middle of the cell are not clearly recognizable in this example. Efferent neurofibrils then leave the cell by the darkly staining axon, five of which are seen in the section. In two of the cells the axon is out of the plane of the section; one of the nerve cells has over-stained, so that details of its neurofibril system are visible only in its dendrites. Numerous weakly staining nerve cells are seen at the left of the section; three blood-vessels are also present. The isolated nuclei are nuclei of neuroglia cells, whose cytoplasm has not stained.

(Bielchowsky reduced silver method.)

But large numbers of these fine fibrils can be traced, sometimes over short, sometimes over very long distances, to the dendrites of the anterior horn cells. At times, if the plane of section has been suitable, these bundles of neurofibrils can be traced back from the dendrites, and can be observed to arise by the convergence of fibrils from various parts of the gray matter (see figure 8, at *x*). At other times the bundles can be traced back from the dendrites over extremely long distances (see figure 6), but eventually pass out of the plane of the section, so that it is not possible to see whence they come. At other times, again, the fibrils which enter the dendrites (in the case of those cells which lie at the edge of the gray matter) can be traced outwards into the white matter (see figure 8).

In the preparations which I have examined, these observations can be made with absolute clearness, and at no time have I ever observed anything of the nature of a discontinuous synapse between these fibrils and the nerve cells. The fibrils penetrate the dendrites, and are now seen to be continuous with, and, indeed, quite indistinguishable from, the neurofibrillae of the nerve cell. No one would maintain that the fine fibrils that pervade the gray matter (outside the nerve cells) are fixation artefacts; it would seem equally erroneous to consider them as artefacts when they are once within the nerve cells. Furthermore, we must surely conclude that the fine fibrils that traverse the substance of the gray matter are conducting elements, probably the actual conducting units, since they arise as collaterals from the neurones that run longitudinally in the white matter. Ought we then to conclude that these fibrils, after they have entered the nerve cells, are no longer conducting elements, but are merely skeletal structures? It must be emphasized that these observations are in no way difficult to make, and provided well-stained material is employed, these facts can be observed with absolute clearness by the use of any high-power objective.

These neurofibrillae, on entering the nerve cell, run, in the dendrites, a perfectly independent course (see figures 6 and 7), and no fusion of neurofibrils can ever definitely be observed. But when the fibrils approach the middle of the cell, and sometimes even when still a considerable distance from it, they appear to branch, and the extraordinary difficulty which we now experience in trying to follow them individually is in marked contrast to the ease with which they may be observed in the outer portions of the nerve cells (see for example, figures 7 and 9). This is due to the convergence of the neurofibrillae in the region immediately around the nucleus, as they enter through the various dendrites, not only those in the plane of section of the nerve cell, but also through all the dendrites that are not in the plane of section. The difficulty is increased by the fact that we are generally observing the neurofibrillae on a very granular background, viz., the cell nucleus.

Despite these facts, however, I can state with considerable confidence that a true anastomosis of fibrils occurs round the nucleus. This observation is in agreement with the work of Cajal (see, for example, figure 75 of Cajal's book),

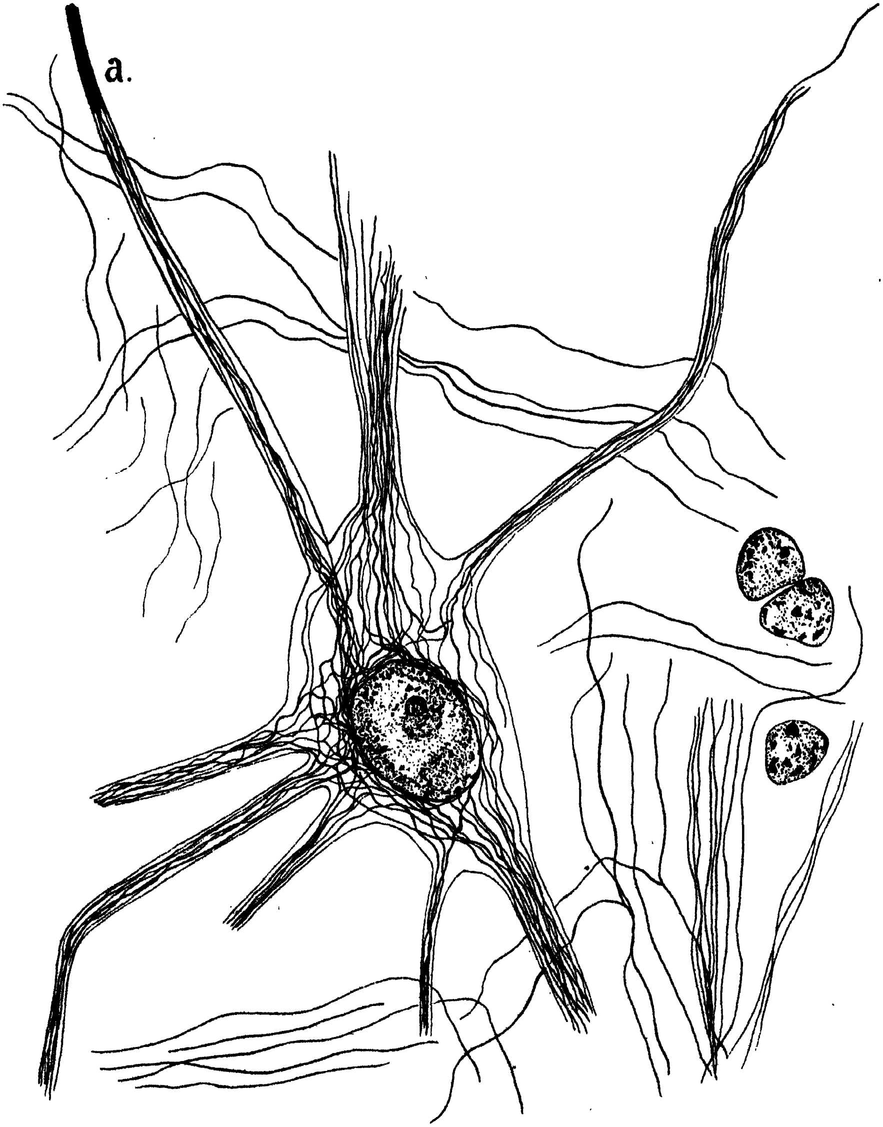


Figure 7. Anterior horn cell (rabbit), with highly developed neurofibril system. The section has been cut along the axon (a) and through the middle of the cell, so that the anastomosing system is not well seen; occasionally, however, some of the neurofibrillae show branching, even in the outer regions of the cell. Note, also, bundles of fibrils (collaterals) and the nuclei of three neuroglia cells in the surrounding extracellular region.

(Bielchowsky reduced silver method.)

and is opposed to that of Bethe. I do believe, however, that the fine meshwork often figured by Cajal as connecting up the whole fibril system is merely an artefact, due to faulty fixation of the cell protoplasm. I have certainly never seen anything resembling it in my preparations, and in this respect am wholly in agreement with Bethe.

The observation that the neurofibrillae form an anastomosing system around the nucleus must, I believe, be regarded as well established; such a condition is seen, for example, in the nerve cell shown in figure 9, where the cell is viewed in a plane immediately above the nucleus (compare in this respect figures 7 and 9). Finally, from this anastomosing system a relatively small number of neurofibrils arise, and leave the cell by the axon.

These observations seem to throw light on the somewhat paradoxical observation of Bethe, that very many of the neurofibrillae enter the nerve cell by one dendrite and apparently leave it by another, without ever entering the axon; for if two neurofibrils, entering the cell by two dendrites, meet an anastomosing system, then clearly the appearance, especially if the section be a thin one, will be that of a single neurofibril traversing the cell from one dendrite to another. I have, indeed, often seen specimens having a close resemblance to those figured by Bethe, but have never been able to observe a case in which the above explanation could be definitely excluded. The two cells shown in figure 8 make this especially clear.

From these observations we conclude, then, that *the fine fibrils that arise as collaterals from the white matter, and pervade the whole of the gray matter, enter the dendrites of the nerve cells, either singly, or more usually in bundles, and without the formation of any discontinuous synapses (this observation has as yet only been made on the anterior horn cells), and that the fibrils (which are now spoken of as neurofibrils), entering the cells by the various dendrites, all converge towards the middle of the cell, where they undergo anastomosis; and that from this anastomosing system a relatively small number of neurofibrils arise and enter the axon.* No details can yet be given concerning the structure of the anastomosing network.

These latter remarks refer only, of course, to the anterior horn cells, so that the question of continuity or discontinuity at neurone junctions in other parts of the nervous system remains to be investigated further.

Concerning the mode of entry of the fibrils into the nerve cells, in my preparations I have never with certainty been able to observe these fibrils entering the *body* of the cell, as described by Bethe and by F. M. Ballantyne, while in every nerve cell that has stained in any way clearly they can be seen entering through the dendrites in very large numbers. I have also never seen anything resembling the Golgi network, described by Bethe as occurring on the outside of the nerve cells.

From these histological observations it seems, then, that we must conclude that the collaterals from the various neurones of the white matter enter the anterior horn cells without the formation of discontinuous synapses, and that the seat of the integration is in the anastomosing system in the deeper parts of the nerve cell. Sherrington's principle of "convergence" seems beautifully illustrated.

But since the neurofibril of the nerve cell, rather than the nerve cell as a whole, must, as we have seen above, be looked upon as the unit of conduction for the nervous impulse, so it seems we must also regard the efferent neurofibrillae that enter the axon as individual conducting units. But why so many efferent neurofibrils, rather than one only, as we find in the leech, should exist we cannot yet definitely state. Nevertheless, the thesis maintained in the foregoing theoretical discussion seems abundantly verified by the histological observations.

SUMMARY.

1. (*a*) From a consideration of the physiology of the organ of hearing, it is concluded that the auditory centre becomes apprised of a gradation in the intensity of the auditory excitation, not by a stimulation of a graded number of auditory neurones, nor yet by a gradation in the frequency of passage of impulses, whether of normal or of subnormal intensity, along these auditory neurones (for the ear is able to register phase difference), but by a gradation in the *magnitude* of the disturbance propagated in the individual neurones.

(*b*) But since the actual process of conduction is one of "all-or-none" intensity, it follows that, underlying this gradation in the magnitude of the response in each neurone, there must occur a gradation in the number of certain elementary conducting paths excited within each neurone.

(*c*) It is then shown that the neurofibrillae are not mere fixation artefacts, for they are capable of being revealed by wholly different methods of technique,

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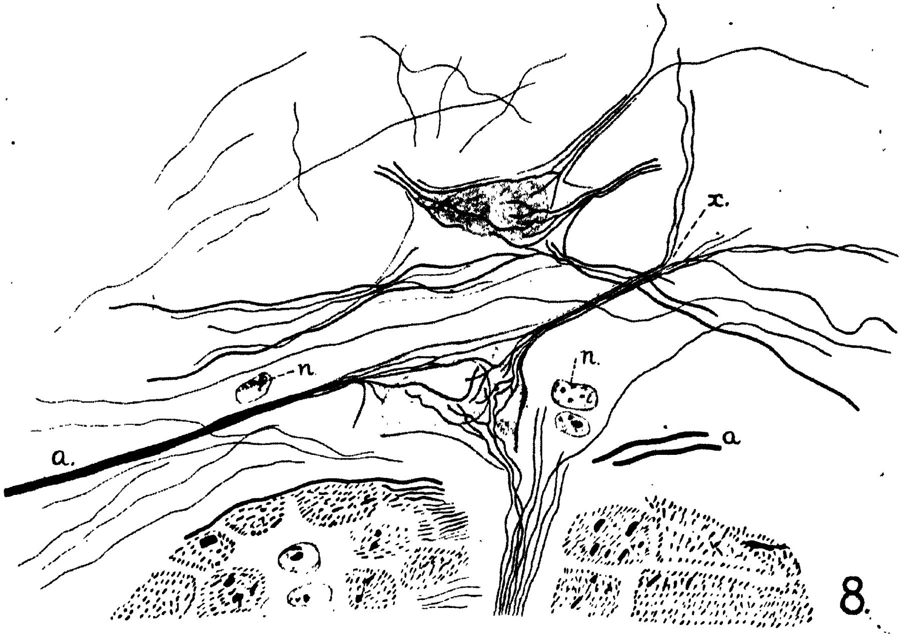
Figure 8. Two anterior horn cells from spinal cord of rabbit, taken from part of the grey matter, closely adjacent to the white matter. In the lower portion of the figure the longitudinally running bundles of nerve fibres of the white matter are seen cut in transverse section. A bundle of fine collaterals is seen, passing from the white matter into the grey; three of these collaterals enter one of the dendrites of the lower anterior horn cell; the others penetrate further into the grey matter, and soon pass out of the plane of the section. Portions of other collaterals are seen running irregularly in the grey matter. Within the nerve cells an indication of the anastomosing system is seen. At x, six fibrils are seen, converging from different regions of the grey matter, to enter a single dendrite.

a, axons; b, nuclei of neuroglia cells.

(Bielchowsky preparation.)

Figure 9. Large nerve cell from anterior horn of spinal cord (rabbit). The section is taken immediately to the side of the nucleus. The anastomosing neurofibril system is clearly seen.

(Bielchowsky preparation.)



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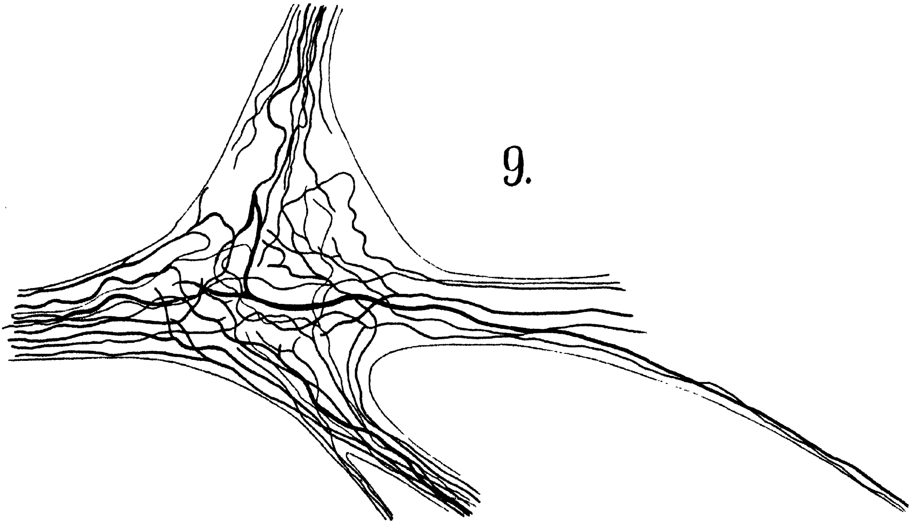

9.

and it is believed that the neurofibrils are the actual conducting units of the neurone. The conclusion is then reached, that the *receptive cells of the cochlea apprise the brain of the intensity of the auditory excitation, by grading the number of neurofibrillae they excite within the individual neurones.*

2. (*a*) From a consideration of motor end-plates as being branched and spreading structures applied in a discontinuous manner to a double helicoidal excitable membrane, it is shown that the absence of secondary conducting paths within these motor end-plates is incompatible with certain accepted laws of tissue excitation. Histologically it is found that the neurofibrils run entirely independent paths within the motor neurone, and that they terminate upon minute "end-knobs," which are in connection with the excitable membranes of the muscle fibre, frequently, perhaps always, with the *two* membranes.

(*b*) It is concluded that reflex excitation of a muscle fibre could be brought about adequately by the stimulation of a single neurofibril in the motor neurone. This is in agreement with the observation, that the directly excited action current of the motor neurone is very much greater than the reflexly excited.

3. Within the spinal cord the neurofibrillae of the sensory nerves separate to form each a collateral, one single afferent neurone thus coming into communication with numerous efferent neurones in the grey matter. This arrangement appears to provide the basis for the gradation of size of reflex response, to gradation in the intensity of stimulation of a peripheral skin-point, in those cases in which central summation seems unable to produce it.

4. The rôle of the many neurofibrils, rather than a single one, in the motor nerves is discussed. On the basis of certain phenomena of reflex action, the suggestion is offered that different types of reflexes which employ the same effectors, but to varying purposes, may act through wholly different final paths, and it seems as if the individual neurofibril may supply such a path. It is pointed out that the apparently primitive arrangement of the neurofibrillae is realized in the leech, whose sensory neurones contain many neurofibrillae, while the motor neurones contain but one.

5. In confirmation of these theoretical conclusions, it is shown histologically that the fine collaterals from the sensory and internuncial neurones traverse the grey matter, and eventually enter the dendrites of the anterior horn cells without the formation of synapses. The various collaterals, entering the anterior horn cells by its numerous dendrites, converge towards the middle of the cell, where they form an anastomosis; it is apparently here that the integration takes place. From this anastomosing system a small number of efferent neurofibrillae arise and enter the axon. In the axon they run an independent course, and eventually terminate individually upon the two excitable membranes of the muscle fibre.

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THE STRUCTURE OF THE NEURONE JUNCTIONS OF THE SPINAL CORD

by

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In the previous paper I have tried to show that the conception of the neurone as the nervous conducting unit is inadequate to explain certain features of the behaviour of the central nervous system, but that such behaviour could be accounted for if we assume the existence of numerous conducting paths within the individual neurones; and it was pointed out that these ultimate conducting units were probably constituted by the neurofibrils. In confirmation of this view it was found histologically that the fine collaterals that traverse the grey matter in the spinal cord enter the dendrites of the anterior horn cells, either singly or in bundles, and become continuous with, and are indeed indistinguishable from, the neurofibrillae within the nerve cell, so that, since the fine collaterals were undoubtedly conducting elements, a conducting function would likewise have to be ascribed to them when they had penetrated the body of the nerve cell, i.e., when they had become identical with the neurofibrils of histologists. It was further pointed out that on the anterior horn cells, synapses, in the form in which they are usually conceived by physiologists, i.e., ramifying, brush-like terminations of neurones, coming into close contiguity, but not into direct continuity with the next neurone of the reflex chain, do not exist. On the contrary, the fine collaterals penetrate the nerve cells, and being now one and the same structure as the neurofibrils, run an independent course in the dendrites till they reach the middle of the cell. Here they appear to undergo anastomosis with the neurofibrillae that enter the nerve cell through its other dendrites, and this central anastomosing system appears to be the seat of the nervous integration.

These observations were obtained by the use mainly of preparations made by the Bielchowsky reduced silver method, and were confined entirely to the anterior horn cells; the posterior horn cells do not usually stain so sharply by this method. Now it has been shown by Sherrington (7) that, assuming the neurone to be the conducting unit of the nervous system, the anterior horn cells are characterized by certain features which distinguish them from all other spinal neurones; they are almost unfatiguable, and their discharge is practically aperiodic. Since fatiguability and periodicity of discharge are regarded as features of synapse action, a simple explanation for this anomalous behaviour of the anterior horn cells might have been forthcoming if discontinuous synapses could have been demonstrated on the posterior horn neurones, or the neurones of Clark's column. But if we assume the terminal neurones to contain many conducting paths, it is unnecessary, as I have pointed out in the previous paper, to ascribe these peculiar properties to the anterior horn cells. The object of the present investigation was to find if any difference could be detected between the termination of neurones on the anterior horn cells, and those of the posterior horn, and of Clark's column. The result of this investigation has been to show that, so far as the microscope can reveal, *the mode of termination of neurones on all the spinal cord cells is the same, and no such thing as a ramifying, discontinuous synapse exists.* It is a curious fact that although numerous descriptions of fibre paths within the spinal cord are to be found in the literature of the subject, a true synapse has rarely been described; only one convincing illustration, so far as I know, has ever been given, namely, a case of several collaterals terminating on three nerve cells of Clark's column (see Cajal (3), vol. I, p. 311, fig. 110).

Methods.

For investigating the neurone junctions of the spinal cord I have used mainly material from very young rabbits (preferably unborn rabbits), though frogs, guinea-pigs, and cats several weeks old have also been employed. In some cases the tissue was stained by the Bielchowsky technique, but the reduced silver method of Cajal (using alcohol as a fixative) is the best process for revealing the structure of the posterior horn cells.

As much of our evidence for the existence of discontinuous synapses rests upon preparations made by the Golgi process, I have taken special pains to verify the results obtained by the reduced silver methods on material prepared by this latter process. I find that the Golgi method, while undoubtedly of inestimable value for tracing out fibre tracts, is entirely useless for revealing the finer details of neurone structure. As a cytological process it is valueless; it causes marked shrinkage of cells, fine precipitates may be deposited irregularly on the fibres and minute fibrils, and of the internal structure of the nerve cell it reveals nothing. While, therefore, the method must always be used as a valuable check on results obtained by the reduced silver process, the latter method must carry much greater weight when minute cytological details are in question.

Since the only convincing figure of a synapse (Cajal's figure) has been drawn from a preparation made by Ehrlich's methylene blue technique, much time has been spent in attempting to verify this observation of Cajal. I find, however, that methylene blue gives precisely the same results as does the Golgi process, and no ramifying, brush-like terminations of fibres embracing the nerve cells can be revealed even by this method. At the same time, methylene blue preparations, made as they are without the aid of any fixatives, present numerous obvious artefacts when microscopically examined. The intercellular portions of the cord tend to become vacuolated, and the nerve cells themselves may at times present a frothy appearance. Obvious coagulation may often be seen on the surface of the nerve cells, and various intermediate stages between such frothing or coagulation, and the apparent ramifying synapses of Cajal may be observed. I find, too, that the superficial Golgi network described by Bethe as occurring on the surface of the nerve cells is to be looked upon as a result of coagulation, for all intermediate stages between such a network and undoubted surface coagulations may readily be observed.

I shall first describe the structure of the neurone junctions of the spinal cord as revealed by the reduced silver methods. Of the two processes usually employed, that due to Cajal and that due to Bielchowsky, the former has been, in my hands, more consistently satisfactory, though the Bielchowsky method, probably on account of its superior mode of fixation, is usually more successful in revealing the finer cytological details.

Anterior Horn Cells.

With the use of the Bielchowsky technique, and employing newborn rabbits and guinea-pigs, I find, as described in the previous paper, that the fine collaterals that arise from the longitudinal fibres of the white matter traverse the grey matter, sometimes in bundles, at other times irregularly alone, and eventually converge from various portions of the cord upon the dendrites of the anterior horn cells, and then penetrate them. Having penetrated the dendrites, they travel along them as the neurofibrils of the cell, and approaching the nuclear region of the cell, undergo anastomosis here. From this anastomosing system a smaller number of neurofibrils arise, which then emerge as the efferent neurofibrillae of the axon. The direct continuity of the neurofibrils of the nerve cell, with the fibrils that constitute the collaterals in the portion of the grey matter outside the nerve cells, seem to be sufficient proof that neurofibrils are not mere fixation artefacts, as maintained by many authors.

Various investigators (Cajal, Held, van Gehucten, Lenhossek, and others) regard the neurofibrils as merely a part of the protoplasmic reticulum of the cell. But as F. M. Ballantyne (1) has pointed out, the explanation is inadequate, for the reticulum may be observed between the fibrils.

Leydig (5), on the other hand, believes that they have a definite existence, but that, nevertheless, the real conducting element of the nerve cell is constituted by the interfibrillar substance. But the necessity for assuming the existence of separate paths within the neurones (8), and the fact that the neurofibrils are continuous with structures outside the cell that are undoubtedly conducting elements, can leave little doubt that the neurofibrillae themselves constitute the conducting elements.

The Bielchowsky process seems, therefore, to show that in the anterior horn cells there is direct neurone continuity between these cells and the fibrils that terminate upon them. In the large number of cases that I have examined such neurone junction always occurs through the dendrites (cat, guinea-pig, rabbit). In these preparations I have never seen anything in the slightest degree resembling branching terminations of neurones ending upon the "body" of the nerve cells; the collaterals *all* enter the dendrites and penetrate to the middle of the cell. I have also never been able to observe the fine collaterals entering the Golgi network on the surface of the nerve cell as described by Bethe (2); while my preparations show neurone continuity with the utmost clearness, such continuity does not occur in the manner described by Bethe. F. M. Ballantyne has observed the neurofibrils directly penetrating the *body* of the nerve cell in *Lepidosiren*. But in the cat, guinea-pig, and rabbit I have never found such a thing to occur; the collaterals all enter through the dendrites. Neurone continuity within the central nervous system has also been described by Marui (6), cited from F. M. Ballantyne (1).

If the anterior horn cells are now examined in preparations stained by the reduced silver process of Cajal, it is frequently found, especially in strongly stained material, that the bundles of fine collaterals that penetrate the dendrites stain a deep black, and it may be difficult, at times impossible, to reveal neurofibrils within them. Nevertheless, even in such preparations, the collaterals can often be seen converging singly upon the dendrites, though it is not possible usually to follow out the individual neurofibrils once they have entered the dendrites, so heavily do the latter stain. But in such preparations, provided that the staining has not been too heavy, it is usually possible to observe neurofibrils again at the point where the dendrites merge into the "body" of the nerve cell. It would seem that these Cajal preparations throw a considerable light upon the misconstruction that has been placed by various investigators upon

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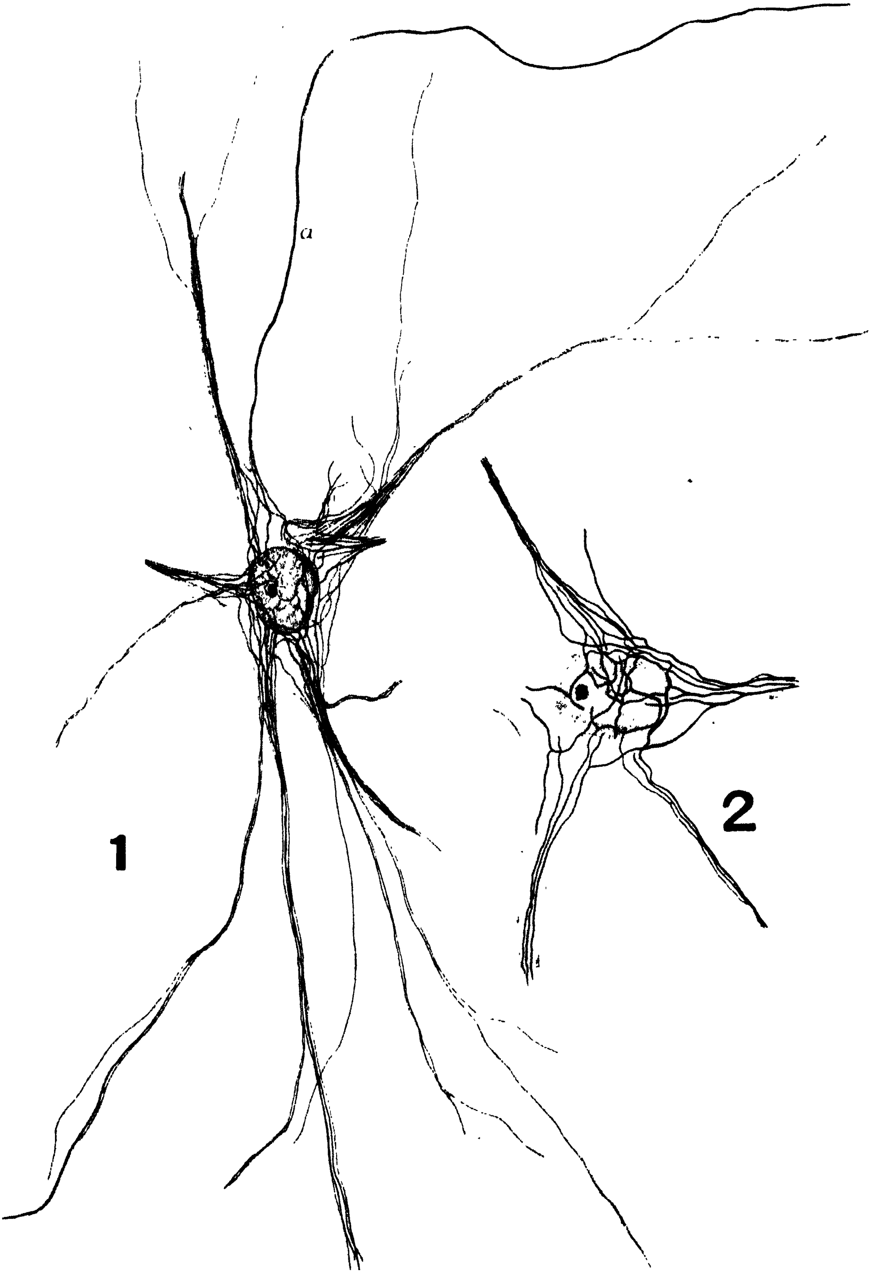
Figure 1. Motor cell from anterior horn of spinal cord (guinea-pig). The dendrites are relatively short structures; into these dendrites run fine fibrils (collaterals from other neurones), and these, retaining their individuality, become identical with the neurofibrillae of the cell. The axon (a) and fibrillae, when traced outwards from the cell, all end sooner or later, but such ending is due to the fact that they emerge from the plane of the section. If this cell had been stained by the Golgi method, it would have stained a uniform black, and the individuality of the collaterals, once they had entered the cell, could no longer have been recognized.

(Cajal's reduced silver method.)

Figure 2. Nerve cell from spinal cord (young rabbit). The focus has been made some distance above the nucleus. The anastomosing network around the nerve cell is evident. Neurofibril discontinuity within the cell is the result of cutting through of the fibrillae.

(Cajal's reduced silver method.)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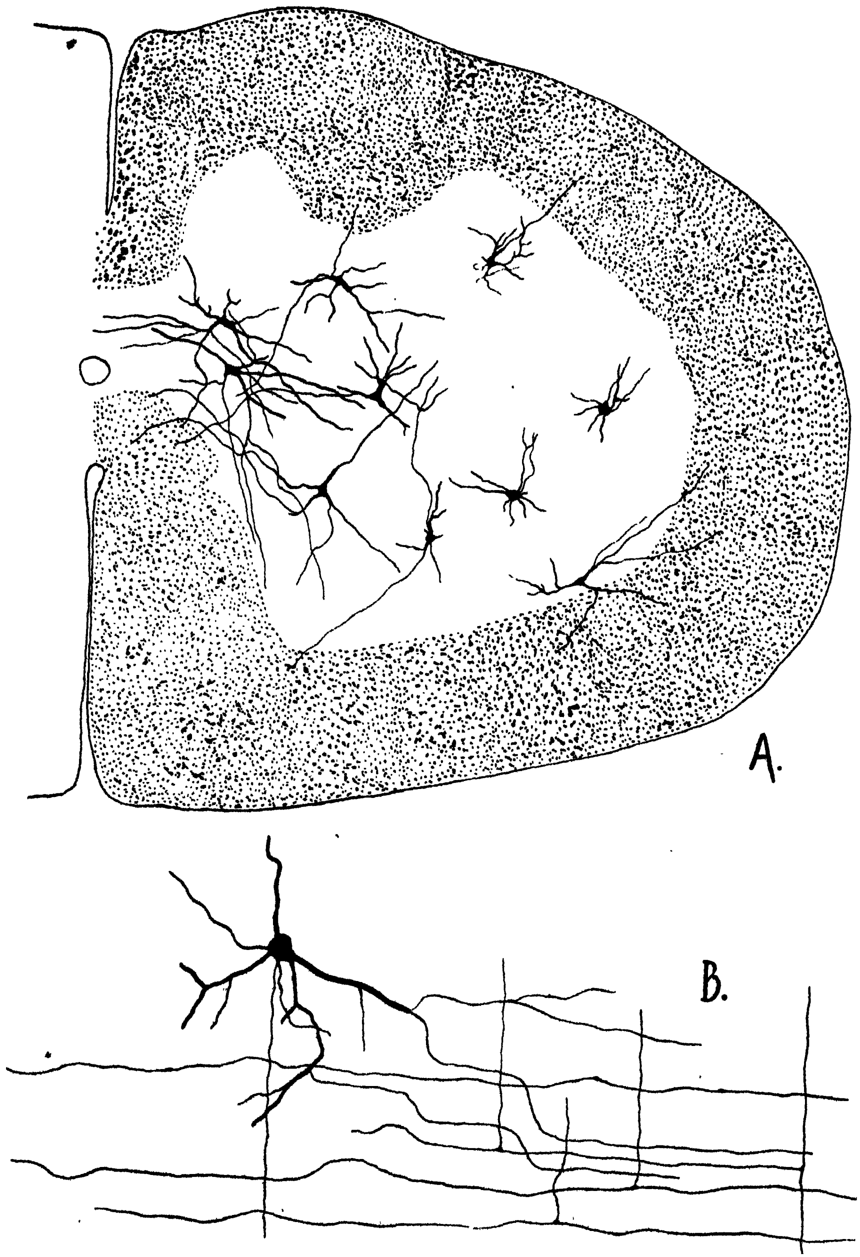
the relation of neurone endings to the following neurone of the reflex chain, for what has been called a dendrite is for its greater length merely a group of neurofibrillae that are approaching the body of the nerve cell in a bundle, and are entering it by way of its dendrites. Since the various technical procedures seem at first sight to yield such diverse results, it will be best to discuss this question first.

If we examine preparations made by Bielchowsky's method, or preparations made by Cajal's process (but not too strongly stained), then, as pointed out above, we can observe neurofibrils converging from various regions of the grey matter, entering the dendrites singly or in bundles, and then, retaining their individuality, approach the middle of the nerve cell (see figure 1). The neurofibrils may be followed, often for incredible distances, outwards from the cells upon which they have converged; eventually, however, they end suddenly, *but such ending is invariably due to the fact that they pass out of the plane of section.* In other words, it is not possible to demonstrate any neurofibril discontinuity in the spinal cord by the reduced silver method; the fibrils merely end because they have been cut through in making the section.

If we now examine the anterior horn cells stained by Golgi's process, an apparently different picture is revealed; the cells appear a homogeneous black, and neurofibrillae are not visible within them. From the "body" of the cells enormously long processes grow out, and these processes in their turn give off long, though very fine, secondary processes (see figure 9). If the sections are thick we can follow the cell processes for very great distances; nevertheless, they all end eventually, but almost always, *because they have been cut short.* (The few cases in which a true termination of the fibrils is observed might readily be due to incomplete staining.) With methylene blue preparations the same can be seen. The numerous figures given in Cajal's book are too well known to need further description. But in describing these anterior horn cells as cells with extremely long dendrites, the fundamental fact is constantly overlooked that the termination of the dendrites is not a real termination, but is merely due to the fact that they must inevitably be cut short as they eventually emerge from the plane of section of the preparation.

The Golgi preparations are accordingly to be interpreted in the following manner: Collaterals, consisting of one or more (?) neurofibrils, enter the grey matter from the white substance of the cord, and make their way towards the anterior horn cells. As they approach nearer the anterior horn cells, delicate fibrils join them, and the bundle grows in thickness; eventually it merges into the relatively short outgrowth from the nerve cell, and this short outgrowth constitutes the real dendrite of the cell. The reduced silver methods reveal the real nature of these neurofibril paths; the Golgi process, on the contrary, failing as it does to render visible any of the inner details of the "dendrites," presents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B.

to us merely the picture of a heavily stained, highly branched cell, and is incapable of showing that the fine fibrils that join the collaterals as they approach the anterior horn cells retain their individuality right through the dendrites into the middle of the cell.

In further criticism of the synapse theory as applied to the *anterior horn cells*, it may be pointed out, firstly, that no convincing case of nerves terminating on the *body* of the nerve cells has ever been depicted. (Cajal's book gives a figure from a motor cell of the medulla, showing numerous minute knobs terminating on the nerve cell. See figure 112 of his book. The illustration is unconvincing, especially in the face of so much evidence leading to the opposite conclusion. The knobs might readily be regarded as endings of neuroglia fibres, for Cajal does not show them to be continuous with nerve fibres. But an even more probable explanation is that they are coagulations, rendered apparent as the nerve cell shrank during fixation. I have seen similar structures, especially with the use of the methylene blue process, and these knobs were certainly not connected with nerve fibres.) Numerous authors, in consequence, are inclined to figure synapses diagrammatically as brush-like terminations of neurones, ramifying among the *dendrites* of nerve cells, rather than terminating in close contact with the body of the cell. For example, the diagrammatic representation of a synapse, as originally presented by Michael Foster (4, figure 97), is partly conceived in this manner. But we should remember that the "dendrites" of a single nerve cell, if traced to their "termination," may ramify over a region occupying sometimes nearly half the area of the complete section of the cord (see, for example, figures 221 and 223 of Cajal's book; the figures refer to the medulla oblongata), and, to be consistent, the brush-like terminations should be of similar dimensions. But it is quite certain that a synapse of this size has never been observed.

It has been pointed out above that in reduced silver preparations the bundle of neurofibrillae that enters a dendrite of a nerve cell may stain a deep black, no internal differentiation into its constituent neurofibrils being visible. In such preparations the neurofibrils that compose the bundle are not visible till

Figure 9. Sections of spinal cord (Golgi method).

A. Transverse section of spinal cord at its upper cervical end (cat). The figure represents a typical Golgi preparation, the nerve cells staining deep black, and possessing many long, at times branching, processes frequently called "dendrites." If the nerve cells of this preparation are compared with the nerve cell shown in figure 1, it will be noted that the "dendrites" of the Golgi preparation are composed of two elements, namely the true dendrites (which are relatively short) and the nerve fibrils (which enter the dendrites); but the Golgi method is not usually capable of rendering the distinction evident. In all cases the "dendrites," when traced outwards, terminate merely because they pass out of the plane of the section.

B. Longitudinal section of part of spinal cord; posterior horn (rabbit). In this preparation the distinction between the dendrite and nerve fibril seems fairly evident, although, of course, even here the fibrils cannot be followed into the cell. Four longitudinal fibres from the white matter are also shown.

the bundle has penetrated the dendrite, when they spread out in the form of a brush. If such a nerve cell were cut in section in such a way that the brush-like expansion of the bundle of neurofibrillae were cut through, the effect would be a structure having a very close resemblance to a branching discontinuous synapse. It is possible that preparations of this kind have lent support to the synapse theory.

We must conclude, then, that the reduced silver methods reveal a true neurone continuity on the anterior horn cells, and the results obtained by the Golgi and Ehrlich methods are not at variance with this conclusion.

Cells of the Posterior Horn and Clark's Column.

When the nerve cells of the posterior horn or of Clark's column are examined, stained by either of the reduced silver methods, precisely the same type of neurone junctions may be observed here, as on the anterior horn cells (see figure 3); if moderately thick sections are examined the fine fibrils may be traced into the cells, sometimes from very long distances. But again we notice, as with the anterior horn cells, that when we trace such fibrils outwards from the cells, they terminate only when they pass out of the plane of the section; again, therefore, no neurone discontinuity can be demonstrated on any of the Clark column or posterior horn cells.

In many preparations it may be observed that the neurones which terminate upon these cells are considerably thicker than a single neurofibril; nevertheless, no fine neurofibrillae can be seen joining them, to which their thickness might have been ascribed. We are therefore not yet able to state whether these fibrils that merge into and are continuous with the nerve cells of the cord are composed always of neurofibrillae that have converged from *various regions of the cord*, or whether they may at times constitute a small bundle of neurofibrils that have all grown out from a single nerve cell.

REFERENCE TO PLATE.

Figure 3. Nerve cell from Clark's column (young rabbit). Note the various nerve fibrils entering the dendrites; the anastomosing network is not recognizable in this preparation. The outer termination of all the fibrils is due always to their leaving the plane of the section of the preparation.

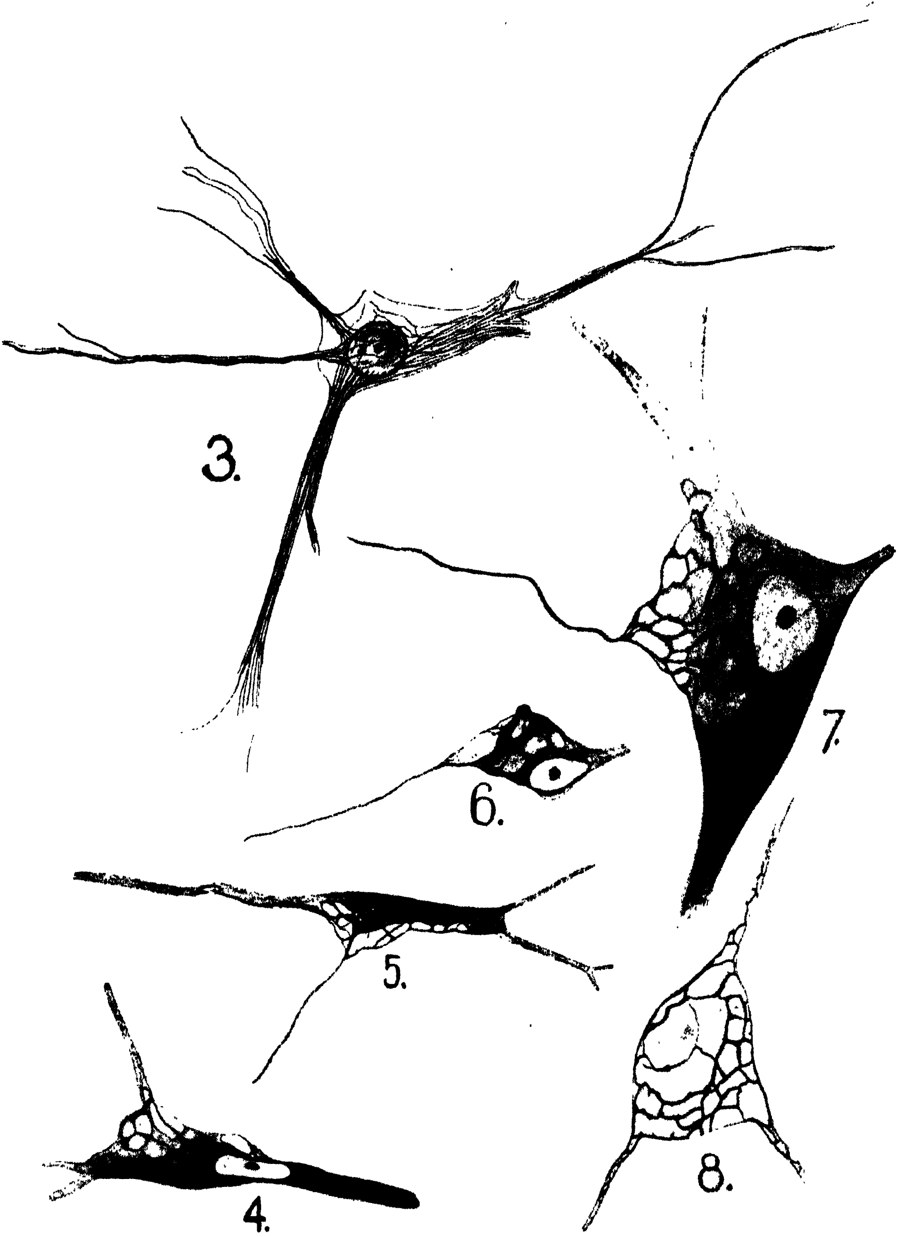
(Cajal's reduced silver method.)

Figures 4, 5, 6, 7. Four nerve cells from spinal cord of frog (*Hyla aurea*). In figures 4 and 6 the conical portion of the cell, which represents the region of outgrowth of a dendrite, has become vacuolated. In figure 5 is shown such a nerve cell, in which the vacuolated dendrite has assumed somewhat the appearance of a ramifying synapse. In figure 7 is shown such a nerve cell, with a highly vacuolated dendrite, giving the appearance of a neurone termination by a ramifying ending, upon the body of a nerve cell.

(Ehrlich's methylene blue method.)

Figure 8. Nerve cell from posterior horn (frog), to show the "superficial Golgi network," an obvious artefact due to coagulation.

(Ehrlich's methylene blue method.)


3.
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4.

Effects Produced by the Metheylene Blue Method.

The main criticism that may be directed against the methylene blue process is that the tissues are never subjected to any process of fixation. We find, consequently, in preparations made by this method that the tissues present numerous obvious artefacts. The gelatinous material occupying so much of the region of the cord between the nerve cells may often present a high degree of vacuolation, and on some of the nerve cells a similar vacuolation may at times be seen. Sometimes, however, the cells stain remarkably clearly, despite the absence of fixation. Now it may be observed in such vacuolated preparations that a single dendrite may contain within it a single large vacuole, or several smaller vacuoles may be present, or a considerable number of very small vacuoles, which give the dendrite a frothy appearance, and such a dendrite may present an extraordinary resemblance to the branching dendrites figured by Cajal as occurring on the cells of Clark's column. It is possible that Cajal's branching dendrites are to be conceived in this light. In figures 4, 5, 6, and 7 a small series of dendrites stained by the methylene blue process are shown, and a gradation from an obviously vacuolated dendrite to one having a close resemblance to the "synapse" of Cajal may readily be observed.

In other cases I find that the surface of the cells presents very obvious coagulation artefacts, and modifications of such surface coagulations may show a marked resemblance to the structures figured by Cajal, Bethe, and others, and called by them the *superficial Golgi network* (figure 8).

The objections that are usually raised against the theory of neurone continuity are, firstly, that transsection of a neurone in a living animal causes degeneration of that neurone along all that portion of the fibre distal to the nucleated part of the cell, the degeneration extending up to but not beyond the next neurone of the reflex chain; secondly, that a reflex arc can conduct in only one direction.

The first objection is a weighty though not an insuperable one. The second does not carry much force, for, even assuming the existence of neurone discontinuity, we are unable to say why such discontinuity should limit the passage of impulses always to the forward direction, unless, of course, we assume the existence of a semipermeable membrane at the termination of such a discontinuous synapse, an arrangement which might equally well be found in a continuous conducting system—continuous, at any rate, so far as the microscope can reveal.

At the same time it seems fairly clear that the processes occurring within the "nerve cells" differ in some respects from those that occur in the fibres. Mosso concluded many years ago that the temperature of nerve cells rises markedly when they function actively, and the rapid fatigue that ensues in nerve cells deprived of oxygen is in marked contrast to the relative absence of fatigue in nerve fibres placed under similar conditions.

From these considerations it seems that we must conclude that the processes which occur within the nerve "cell" during excitation, are not entirely similar to those which occur within the nerve trunks, and when such special properties of the nerve cell can be demonstrated, it would seem that the property of forward conduction of the reflex arc could be attributed to these, and that a visible discontinuity in the conducting system need not be postulated.

SUMMARY.

1. By means of the reduced silver methods of Bielchowsky and of Cajal, it is found that the fine collaterals that enter the grey matter from the white matter of the spinal cord converge upon the nerve cells of the grey matter, and penetrate the dendrites of the nerve cells, being continuous with, and indeed quite indistinguishable from, the neurofibrils of the nerve cell. These fine collaterals, having entered the nerve cells, pass independently of one another, as the neurofibrils, towards the middle of the nerve cell, where they anastomose. From this anastomosing system a small number of neurofibrils arise, and emerge through the axon.

2. The anastomosing system appears to be the seat of the integration, with the component phenomena of summation, inhibition, fatigue, delay, one-directional conduction, etc.

3. It is shown that preparations made by the methods of Golgi and of Ehrlich lead to similar results, much obscured by the fact, however, that they do not usually render visible the internal differentiation of the nerve cells and dendrites. Dendrites are relatively short structures, and the greater portion of the enormous "dendrites" made apparent by the Golgi method are in reality bundles of neurofibrillae, whose real nature is obscured through a defect in the method of staining.

4. Ramifying synapses, coming into close contiguity, but not into direct continuity with the nerve cells, do not seem to occur in the spinal cord. It is suggested that the ramifying synapses of Cajal, and the superficial Golgi network of Bethe, are artefacts due to coagulation and frothing of the protoplasm.

5. It is concluded, therefore, that a true neurone continuity occurs at the junction of neurones in the spinal cord, but such neurone continuity is not a dendrite continuity of the type originally described by Gerlach, nor a continuity through neurone collaterals of the type upheld by Golgi and Nansen, nor yet a continuity through a superficial Golgi network, as maintained by Bethe, but a continuity in which collaterals from various neurones penetrate the various dendrites, and, passing into the substance of the nerve cells, anastomose, the anastomosis with collaterals from other neurones thus permitting evidently of integration, and the whole of the integration occurring within the body of the nerve cell.

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STUDIES ON THE REFRIGERATION OF MEAT

II. THE FREEZING OF BEEF AND MUTTON PRESS JUICES.

by

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(Submitted for publication 3rd May, 1926.)

1. INTRODUCTION.

In a previous paper (2) experiments on various factors affecting the degree of reversibility of beef during the freezing and thawing cycle were described. In these experiments one of the most important factors was found to be the rate of freezing, but complete reversibility, as measured by the amount of drip exuding from a cut surface, was obtained only when freezing was almost instantaneous, liquid air being used as the freezing medium. In all other experiments, when the latent period of freezing was reduced to as low as 0·4 hour, there was still an appreciable quantity of drip on thawing. In the refrigeration of animal muscle changes may be produced both in the contents of the muscle fibre (muscle plasma) and in the surrounding membrane (the sarcolemma), and alterations in the latter may bring about changes in its permeability. The presence of drip in beef after freezing and thawing may therefore be due to irreversible changes either in the plasma or in the sarcolemma, or in both. With the hope of throwing further light on the problem, experiments have been carried out with the juice expressed from minced muscle. Although the colloidal system within the fibre is non-homogeneous, the juice obtained in the above manner approximates to the muscle fibre contents.

Press-juice from ox muscle was used in most of these experiments, but comparative tests were also made with mutton press-juice, to ascertain if the well-known differences in the properties of beef and mutton during freezing and subsequent thawing, are due to differences in the behaviour of the muscle plasma.

2. EXPERIMENTAL METHODS.

(a) Preparation of the Press-juice.

Attempts were made to prepare a sample of juice from the muscle before rigor mortis had set in, as it was thought that a more representative sample could be obtained in this way. Owing to the fact that the whole muscle was in a jelly-like state immediately after death, it was found impossible to express any appreciable quantity of juice, 60 cc. being the maximum amount obtained from 6 kgm. of meat. In all experiments, therefore, juice was obtained about 48 hours after rigor mortis had passed off.

The muscle was freed from fat and connective tissue, finely minced, and ground with clean, fine sand. The pasty mass was then pressed in a filter press, and a viscous, clear, red liquid was obtained. This liquid was centrifuged for twenty minutes at about 2,000 revolutions per minute. The total nitrogen content of the supernatant liquid was determined in 5 cc. by Kjeldahl's method. This was found to vary in the several series from 1·30 to 1·80 gm. per 100 cc. of juice. A few drops of toluene were added to prevent bacterial action.

(b) Freezing.

As soon as possible after centrifuging, definite volumes of the juice were frozen in uniform cylindrical glass vessels, using as the freezing media either air, a saturated solution of calcium chloride, or mercury, at temperatures which were arranged to give the desired rate of freezing. Different rates of freezing were obtained also by using cylindrical vessels of different diameters in the same medium. Each vessel was fitted with a thermo-junction (either glass-covered or bare), held in position in the centre of the juice by corks, as shown in figure 1. This gave the minimum rate of freezing for the sample of juice, and the cork stoppers prevented evaporation during storage in the frozen condition. Supercooling was obviated by gently shaking the contents of each vessel every few minutes.

(c) Thawing.

Thawing of the frozen juice was carried out either in air or in water, so that the rate could be regulated to give very fast or very slow thawing. When thawing was completed, 1 cc. of each sample was removed for ultramicroscopic examination. The juice was immediately centrifuged under the same conditions as were previously used. This process precipitated all the small "dehydrated" masses which were suspended in the juice. Since most of the colloidal material consisted of protein and their derivatives, it was safe to assume that the precipitated material was mainly nitrogenous in character. The total nitrogen of the supernatant liquid was then estimated in 5 cc. as before. The greater the degree of reversibility, the greater will be the total nitrogen content (i.e., the total soluble protein) of this supernatant liquid, complete reversibility being shown when the nitrogen content of the supernatant liquid is equal to the content before freezing. The results have been expressed as the ratio of the total nitrogen content of the supernatant liquid after freezing and thawing to the total nitrogen content of the fresh juice.

(d) Examination with the Ultra-microscope.

It was suggested that if actual ultramicroscopic examination of the juice were carried out, both before freezing and after thawing, an indication might be obtained as to the extent of the coagulation, if any, taking place during the freezing and thawing cycle. For these observations the ultramicroscopic apparatus of Zeiss was used in conjunction with a cardioid condenser. Since the numbers of particles observed in a given field will vary with the degree of illumination, after setting up the apparatus and adjusting the illumination to a convenient strength, it was not disturbed until all the experiments were completed. It was assumed that slow freezing would cause an aggregation of

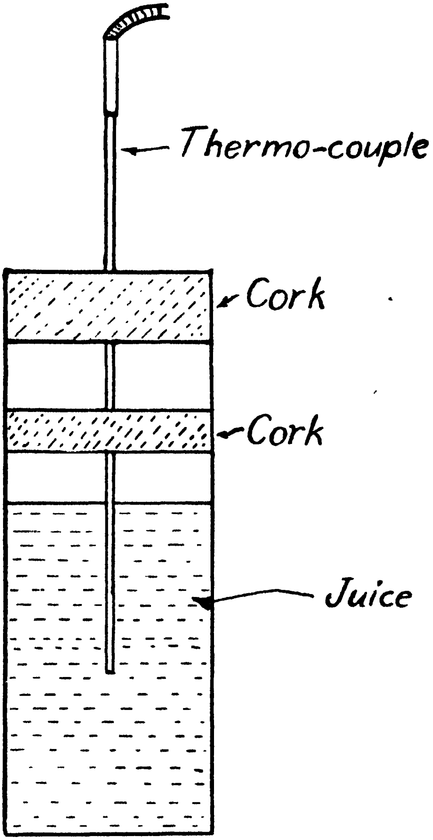

Figure 1.

the single particles, and the slower the freezing the less would be the number of single particles remaining in the fluid. Hence, a comparison was made by counting the actual numbers of single colloidal particles possessing marked Brownian movement, contained in a given volume before and after freezing and thawing. This method was open to criticism, since, in some cases, freezing may cause an actual disappearance of large numbers of single particles which may form aggregates containing two or three. These aggregates, still possessing strong Brownian movement, would not separate out when the juice was centrifuged. Hence, although there would be a distinct diminution in the numbers of single particles remaining, the degree of reversibility, as measured by the first method, would be great. Unfortunately, no more satisfactory method of estimating the degree of reversibility could be found. The ultramicroscopic examination was carried out in the case of one series only.

3. RESULTS.

(a) Changes in Nitrogen Content.

In the frozen condition, the samples of juice which had been slowly frozen in air showed numbers of fairly clear, wide ice crystals stretching from the sides of the cylinders. On the other hand, the samples which had been frozen more quickly, for example, in calcium chloride, showed numerous, very small crystals scattered throughout the solid mass. After thawing had been completed the more slowly frozen samples had extensive brownish-coloured precipitates of "dehydrated" protein, with small particles suspended in the liquid, whilst the faster frozen juices had small precipitates. In both cases these precipitates appeared immediately after the frozen system had begun to liquify. Stiles (1) states that this flocculation is exhibited by many colloidal solutions which have been frozen and thawed, such solutions showing numerous flocks, which gradually precipitate after thawing.

TABLE 1.

		Freezing			Thawing.			Ratio.
Expt.	Diam. of Cylinder. cm.	Medium.	Temp. Degrees C.	Latent Period. Hours.	Medium.	Temp. Degrees C.	Latent Period Hours.	$\frac{a_1}{a_2}$
1	3·5	Air	—12·5	3·5	Water	20	0·25	0·76
Beef Juice	3·5	Air	—12·5	3·5	Air	21·5	1·1	0·84
75 cc.	3·5	$CaCl_2$Aq.	—12	0·5	Water	20	0·25	0·95
Samples	3·5	$CaCl_2$Aq.	—12	0·5	Air	21·5	1·1	0·96
2	3·5	Air	—14	3·0	Air	13	2·1	0·95
Beef Juice	3·5	Air	—14	3·0	Air	1	9·5	0·95
50 cc.	3·5	Mercury	—4	1·1	Air	13	2·1	0·96
Samples	3·5	Mercury	—4	1·1	Air	1	9·5	0·95
	3·5	$CaCl_2$Aq.	—14	0·3	Air	13	2·1	0·96
	3·5	$CaCl_2$Aq.	—14	0·3	Air	1	9·5	0·95

			Freezing.			Thawing.		Ratio.
Expt.	Diam. of Cylinder. cm.	Medium.	Temp. Degrees C.	Latent Period. Hours.	Medium.	Temp. Degrees C.	Latent Period Hours.	$\frac{a_1}{a_2}$
3	3.5	Air	—10	3.0	Air	13	2.1	0.95
Mutton	3.5	Air	—10	3.0	Air	1	9.5	0.94
Juice	3.5	Mercury	—4	1.1	Air	13	2.1	0.96
50 cc.	3.5	$CaCl_2Aq.$	—14	0.3	Air	13	2.1	0.96
4	7.0	Air	—12	5.4	Water	7.5	0.5	0.84
Beef Juice	7.0	Air	—12	5.4	Air	4	—	0.90
50 cc.	3.5	Air	—12	3.9	Water	7.5	0.35	0.88
	3.5	Air	—12	3.9	Air	4	—	0.92
	3.5	$CaCl_2Aq.$	—13.8	0.35	Water	7.5	0.35	0.92

The results of this method of testing reversibility are given in Table 1, which gives the conditions of freezing and thawing to which the juices have been subjected. The last column gives the ratio of the percentage of nitrogen in the thawed supernatant liquid (a_1) to the percentage in the fresh juice (a_2).

These results showed that the maximum reconstitution of the beef juice attainable in these experiments was 96%, which value was observed with latent periods of freezing up to three hours. For these latent periods the reconstitution is not affected by the rate of thawing. On the other hand, in the experiments where this latent period was exceeded (experiments 1 and 4), reconstitution was aided by slower thawing. In experiments 2 and 3, in which comparisons were made between the juices from beef and mutton, for latent periods of freezing up to three hours, no difference was observed, both yielding the greatest degree of reversibility which it was apparently possible to attain.

(b) Examination with the Ultramicroscope.

This procedure was used in the cases of experiments 2 and 3, Table 1. An eye-piece was employed with a field divided into squares, and the numbers of single colloidal particles in one square were counted. Since the same depth (2μ) of fluid was always used, the results were strictly comparable. The results were expressed (in Table 2) as the ratio $\frac{A_2}{A_1}$ of the number of single particles in a given volume of thawed juice (A_2) to the number in the same volume of fresh juice (A_1). Fifty counts were made in each experiment, and the average values recorded. The samples of fresh beef and mutton juices contained an average of 6·65 and 11·70 single particles respectively in the given volume examined. These figures refer to the term A_1 in the ratio.

TABLE 2.

Juice.	Latent Periods. Freezing. Hours.	Latent Periods. Thawing. Hours.	A_2. Number of single particles in vol. x of thawed juice.	Ratio. $\frac{a_1}{a_2}$
Thawed Beef Juice	3.0	2.1	3.2	0.48
” ” ”	3.0	9.5	3.8	0.57
” ” ”	1.1	2.1	3.8	0.57
” ” ”	1.1	9.5	5.1	0.76
” ” ”	0.3	2.1	4.3	0.64
” ” ”	0.3	9.5	5.6	0.84
Thawed Mutton Juice ..	3.0	2.1	10.7	0.90
” ” ”	3.0	9.5	11.0	0.93
” ” ”	1.1	2.1	10.4	0.88
” ” ”	0.3	2.1	11.0	0.93

The slower freezing of beef juice caused quite a considerable number of single particles to disappear. The smaller number of particles probably indicated the formation of aggregates too small to be precipitated by centrifuging, since the figures for the nitrogen contents quoted in Table 1 showed that almost perfect reconstitution had been attained in all the experiments having a latent period of 3 hours and under. Even in the beef juice having a latent period of freezing of 0·3 hours, considerable numbers had disappeared. Slow thawing was found to have allowed time for a greater redispersion of particles.

In contrast to beef juice, mutton juice showed very little disappearance of particles, even with latent periods of freezing up to 3 hours. This result may be attributed to greater difficulty in redispersing the beef colloid aggregates formed on freezing, owing possibly to a greater degree of "dehydration" taking place in the colloidal particles of beef juice.

4. DISCUSSION.

In a review of the process of the freezing of tissues, Plank (4) stressed the importance of two effects of freezing, viz., the damage to the protoplasmic structure by the formation of ice crystals, and the consequent "dehydration" of colloid which is entailed. He also pointed out the importance of what he considered to be the two main factors in determining whether reconstitution of the protoplasm would take place, viz., the rate of freezing and the temperature to which the frozen substance is cooled. If the results of the experiments on the freezing and thawing of beef juice can be applied to the muscle plasma, they tend to show that Plank has omitted one very important factor in his theory of the freezing of muscle tissue, viz., the properties of the sarcolemma during freezing and thawing. Practically complete reversibility on thawing may be obtained by freezing beef juice at such a rate as to give a latent period of 3·0 hours; with the tissue as a whole (plasma and sarcolemma) the same degree of reversibility is not attained until the latent period of freezing has been reduced to at least 0·5 hours. Hence, it would seem as if the sarcolemma plays a very important rôle in determining the amount of reconstitution which is to take place. Possibly an increased permeability of the sarcolemma takes place on exposure to temperatures of 0°C. and below, for Foster and Moyle (3) state that such increased permeability would account for the loss of irritability which takes place when frogs' muscles are exposed to a temperature of 0°C. for a long period. The slight differences between beef press-juice and mutton press-juice on freezing at a speed which would give a latent period of 3·0 hours, seem insufficient to account for the differences in the properties of beef and mutton during freezing, and suggest that the difference lies to a considerable extent in the changes of the sarcolemma.

SUMMARY.

1. The determination of the amounts of unprecipitated nitrogen before freezing and after thawing, showed that fairly complete reconstitution of beef juice was attained with rates of freezing which gave latent periods of 3 hours and under.

2. This reconstitution was aided by slow thawing.

3. Very little difference between beef and mutton juices could be detected by this nitrogen content method.

4. Ultramicroscopic examination of beef juice showed that the freezing and thawing cycle had caused large reduction in the numbers of single colloidal particles. The higher the rate of freezing, however, the less was the reduction.

5. Mutton press-juice, unlike beef juice, showed very little diminution in the numbers of single colloidal particles, but the variation of the rates of freezing from 0·3 hour to 3 hours did not produce any material alteration in the numbers.

6. The results of the two methods may be reconciled by supposing that during the freezing process the colloidal particles of beef juice may have formed small aggregates, which would not be precipitated by centrifuging.

7. The results suggest that changes which take place during the freezing of beef are largely determined by alterations in the sarcolemma of the muscle during freezing.

8. The differences between the properties of beef and mutton during freezing and thawing may be partly due to differences in the properties of their respective sarcolemma.

In conclusion, I desire to thank Associate Professor W. J. Young for his many helpful suggestions and constant interest in the investigation, and Mr. G. A. Cook, of the Commonwealth Institute of Science and Industry, for performing many of the physical measurements.

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STUDIES ON THE MITOCHONDRIA OF PARAMOECIUM

by

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(Submitted for publication 7th May, 1926.)

The investigation reported in this paper was directed towards determining the behaviour of the mitochondria of Paramoecium during the life cycle of the organism, with a view to helping to elucidate the question of the nature of these bodies and the part they seem to play in the life of the cell. The results obtained in this research seem to show (a) that the mitochondria are true cytoplasmic bodies, and not merely coagulations of the cell protoplasm following upon fixation; (b) that no evidence is yet available to show that they are formed *de novo* from the cytoplasm, but that on the contrary they always increase in number by binary fission; (c) that the occurrence of fission in the mitochondria is irregular, and is quite independent of the occurrence of fission or conjugation in the organism; (d) that they are concerned, on the contrary, with cell metabolism, playing an important part in the process of intracellular digestion (this latter observation will serve to correlate our knowledge of the function of the mitochondria in a unicellular organism, and in the cells of higher animals, where they give rise to extracellular enzymes): (e) taking into account the theory that they are enzymatic in nature, and my previous observations on *Opalina*, where they appear to be the centres of protoplasmic synthesis within the cell, their behaviour appears to illustrate the capacity of enzymes to reverse their activity in accordance with conditions, i.e., synthesis or hydrolysis, according to concentration of the substrate.

Methods of Investigation.

For rendering visible the mitochondria of Paramoecium, the janus green vital stain may be employed; or organisms, fixed in osmo-chromic fluid, may be cut in section and stained with iron haematoxylin.

The former method is particularly useful in dealing with cases in which only a single individual of a thick culture is in the particular stage of the life cycle of the organism that is at the time required for investigation, such, for example, as the conjugating phase, which occurs only very rarely. It is practically impossible to treat such isolated individuals by the haematoxylin method.

The second method yields much more illuminating cytological results. A pure culture of Paramoecia, grown in a solution of malted milk (1 gram per litre), is fixed with the osmo-chromic mixture, or in Flemming solution (omitting the acetic acid). After fixing, the culture is centrifuged and the fluid poured off. By this means the Paramoecia are passed through alcohol, cleared, and eventually embedded *en masse* in paraffin, and then sections cut, which are subsequently stained with Haidenhein's process. When very large numbers of individuals, prepared by this method, are examined, organisms in the various stages of the life cycle are readily recognized, and their mitochondria may be studied at leisure. By this means I have examined the mitochondria during all stages of the life cycle, except the conjugation phase, which I have only studied in living specimens stained with Janus green.

The greatest source of error in mitochondrial work is that it is usually very difficult to distinguish mitochondria from rod-shaped bacteria. But a ready test is available: mitochondria fixed for 24 hours in absolute alcohol are not capable of subsequent staining with iron haematoxylin; bacteria, on the contrary, are not visibly affected by this treatment with alcohol. The mitochondria do not entirely dissolve when placed in alcohol, for they can often be clearly seen in sections made from such material, but they are incapable of being stained by haematoxylin.

DESCRIPTION OF PLATE.

All figures (except fig. 9) are drawn from osmo-chromic material, stained with iron haematoxylin.

Fig. 1. Longitudinal section of adult Paramoecium, showing organization of the mitochondria. Mitochondria near the surface of the organism are shown dark; those in the deeper part of the organism paler. Note the arrangement of the superficial mitochondria in longitudinal rows. Meganucleus and micronucleus clearly visible.

Fig. 2. Transverse section of an adult Paramoecium, containing six food vacuoles. Note the large food globules within the vacuoles. Note also the surface arrangement of the mitochondria; the mitochondria in the deeper parts of the organism are seen entering the vacuoles. Some of the vacuoles contain numerous mitochondrial bodies (not bacteria).

Fig. 3. Longitudinal section of same, showing five foot vacuoles containing numerous mitochondria (not bacteria). Note also mega- and micro-nucleus.

Fig. 4. Paramoecium undergoing binary fission.

Fig. 5. Paramoecium, showing commencement of fission of micronucleus. The focus is along the middle of the organism.

In figs. 5 and 6 it is seen that although many of the mitochondria are in the dumb-bell shaped condition, the proportion of dividing to non-dividing mitochondria is not greater than usual; micronuclear division is not, therefore, accompanied by a general mitochondrial fission.

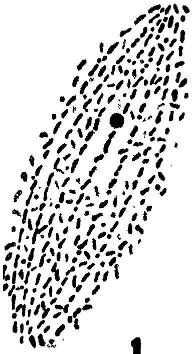
Fig. 6. The same; in surface view.

Fig. 7. An encysting Paramoecium, centrifuged for 1¾ hours before fixation. Note separation of mitochondria and nucleus.

Fig. 8. A normal cyst, not centrifuged.

Fig. 9. Two Paramoecia undergoing conjugation. Taken from two living individuals, stained with janus green (vital stain).

Fig. 10. Young Paramoecium, taken after fission.



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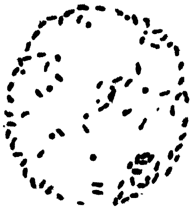

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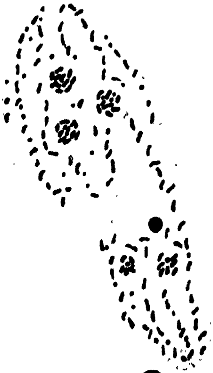

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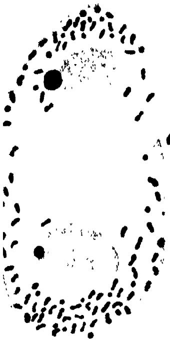

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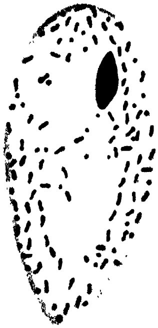

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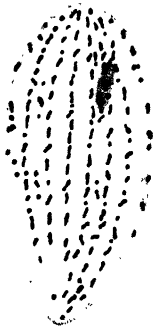

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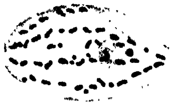

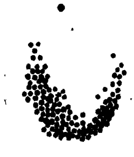

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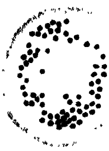

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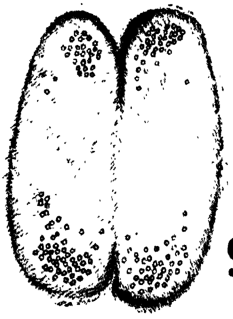

9

The Mitochondria of the Adult Paramoecium.

When Paramoecia, cut in longitudinal section and stained with haematoxylin, are examined under high magnifications, the cytoplasm is found to contain a large number of rod-shaped bodies. If the organism is examined in its deeper parts, these rod-shaped bodies are seen to be disposed somewhat irregularly, and no distinct polarity is recognizable; but if the outer region of the organism is observed, it is found that these bodies are arranged one behind the other in rows, stretching right along the length of the organism (figure 1).

These bacteria-like bodies are the mitochondria. This statement is based on the following facts: (a) Similar bodies may be observed in the living organism when stained by Janus green; (b) Paramoecia fixed in absolute alcohol appear entirely devoid of these bodies; (c) when Paramoecia encyst naturally, or when encystment is induced in them by placing them in Ringer's fluid, the rod-shaped bodies as such disappear, and transform themselves into spherical bodies in precisely the same way as do the mitochondria of *Opalina* (5), and the mitochondria of the pancreas (4); (d) many of the mitochondria are undergoing, as will be described below, transverse binary fission of a kind similar to that observed in the mitochondria of *Opalina.* From these observations the conclusion is drawn that the rod-shaped bodies present in the cytoplasm in Paramoecium are identical with the bodies usually referred to as mitochondria.

Before describing the behaviour of the mitochondria during the life cycle it is necessary to draw special attention to the fact that we possess the very strongest evidence that these bodies are *genuine cytoplasmic inclusions, and not merely artefacts due to the action of the fixing fluid.* We have as evidence for this statement the following facts: (a) Highly refractive bodies, having a similar appearance to the mitochondria, may be observed, though not very distinctly, in living Paramoecia, if these are examined under high magnifications; (b) janus green is capable of staining these bodies in the living organism at a time when the cell protoplasm has not yet coagulated; (c) if a culture of living Paramoecia is strongly centrifuged for about 1¾ hours, the organisms gradually encyst; if such cysts, fixed in osmo-chromic, are now examined and compared with cysts that have been centrifuged only subsequent to fixation, it is found that in the former the mitochondria have largely separated from the rest of the cell protoplasm, and have accumulated at one end of the cyst, while the nucleus and micronucleus lie in a mass of clear, granule-free cytoplasm at the other (figure 7). It is obvious that in this case the granules can be separated from the rest of the cytoplasm, and when this cytoplasm is now coagulated with fixatives, it does not exhibit any granulation. A similar observation has been made by E. N. Browne (2) in the spermatocyte cell of *Notonecta.*

The Behaviour of the Mitochondria during the Life Cycle.

If very small individuals in a preparation made by the above method are examined—Paramoecia, that is, which have just been formed by the binary fission of the adult organism—it is found that the mitochondria have already the typical appearance as seen in the adult Paramoecium, i.e., they are rod-shaped bodies, longitudinally disposed in rows, on the more superficial parts of the protoplasm, but somewhat irregularly arranged in the deeper parts (figure 1). But we always observe in such organisms that the mitochondria are present in very small numbers.

As the Paramoecium grows, the number of mitochondria increases greatly, and such increase is evidently due to a binary transverse fission of the mitochondria, for many of the mitochondria may be observed in these growing organisms, in a dumb-bell shaped condition, in which they are evidently dividing into two parts. Eventually the adult condition shown in figure 1 is reached. There is no evidence to show that fission occurs simultaneously throughout all mitochondria in the Paramoecium; on the contrary, it occurs quite irregularly, and appears to be an inherent property of the mitochondria themselves, rather than the result of a force acting throughout the whole of the organism.

No evidence has yet been obtained to show that the mitochondria might possibly be formed *de novo* from the cytoplasm. It would, of course, be extremely difficult to exclude this possibility, but so far as I have been able to observe, they arise only by the fission of other mitochondria.

When the adult Paramoecium undergoes binary fission, the mitochondria merely pass, without undergoing fission, into the two daughter cells. Fauré-Fremiet has stated (3) that fission of the micronucleus is accompanied by a fission of the mitochondria, the rod-shaped bodies all undergoing fission. recognizable by their dumb-bell shape. But I find that in Paramoecium such a thing does not occur; many of the mitochondria are in the dumb-bell shape form, but the proportion of such dividing mitochondria is not greater than that found in the adult Paramoecium. The fact that the number of mitochondria in the small daughter cell is so very much less than that occurring in the adults prior to micronuclear fission is further evidence against the statements of Fauré-Fremiet.

The behaviour of the mitochondria in conjugating organisms, I have observed only in the living animal, stained alive with janus green. So far as I could observe, no interchange of mitochondria take place during conjugation, and the mitochondria do not seem to take any part whatsoever in the process (figure 9).

When the Paramoecium undergoes natural encystment, or when encystment is induced by placing them in Ringer's fluid, the mitochondria round themselves off, and are seen in the fixed cysts as numerous minute, strongly-staining, spherical bodies, which apparently elongate again, when the organism once more assumes the free, swimming condition.

These observation seem to show, then, *that the behaviour of the mitochondria during the life cycle of the Paramoecium is in no way related to any genetic or sexual phenomena during the life cycle, so that it would rather suggest that the mitochondria were concerned with the general metabolism of the cell.*

The Function of Mitochondria in the Metabolism of the Cell.

There are a number of very significant observations in existence which seem to demonstrate that the mitochondria are bodies having an enzymatic nature. In 1909 Regaud and Mawas suggested that in the salivary glands the secretory granules arose from the mitochondria, thus implying their relationship to enzymes, while Arnold (1) observed the origin of the zymogen grains in the pancreas from mitochondria. Marston (6) has pointed ou that the behaviour of mitochondria towards azine dyestuffs is identical with the reactions which proteolytic enzymes undergo in the presence of these substances, and concludes that they may be the synthesizing enzymes within the cell, their function being to build up protein at a water-poor phase at their surface. In agreement with this I find that in the pancreas the zymogen grains, which evidently constitute the tryptic secretion, arise from the filamentous mitochondria, being simply constricted off from these (4), while in *Opalina* I find (5) that in close connection with each mitochondrium a large granule may develop, this granule being evidently the result of the local synthetic activity of the mitochondrium, formed as it is by the concentration of highly diffusible foodstuffs that have penetrated the cuticle of the organism.

In Paramoecium this apparent synthetic activity of the mitochondria cannot be detected with the microscope—possibly *Opalina* is a very favourable object for the investigation of this point. But in Paramoecium, feeding as it does in the true holozoic manner, a process of intracellular digestion, very similar to the process of extracellular digestion observed in gland cells, can apparently be readily demonstrated. If Paramoecium from an actively feeding culture, living in an extract of malted milk, are examined, the food vacuoles are usually seen to contain a large, rounded globule, this being evidently the coagulated milk from the culture fluid; but a very striking feature of such preparations is that these globules frequently contain a number of rod-shaped bodies, closely resembling bacteria, and also having the same appearance as the mitochondria (figure 3). They are not present in all the individuals of a culture; in one culture, for example, I found them in 76% of the individuals; in another culture only about 31%. The possibility that these bodies might be bacteria, engulfed in the food, or even symbiotic bacteria, seems excluded by the following two considerations: I find that they are present in the food vacuoles of Paramoecia reared in culture fluid that was entirely devoid of bacteria; secondly, if such Paramoecia are killed in absolute alcohol, and then stained with iron haematoxylin subsequent to sectioning, these rod-shaped bodies within the vacuoles do not stain, though they are readily observed in controls prepared from the same culture and fixed in an osmo-chromic fixative. Thirdly, they are stainable by safranin, after osmo-chromic fixation, but alcohol fixation deprives them of their capacity for staining. These observations, and their obvious resemblance to the mitochondria of the cell protoplasm, are taken as strong evidence that these bodies are identical with the latter, a conclusion which is supported by the fact that some of them are in the dumb-bell shaped condition. In *Carachesium,* Fauré-Fremiet has observed that the mitochondria tend to cluster around the food vacuoles. Within the vacuoles these bodies seem gradually to disintegrate; some of them certainly have a fragmentary appearance. It is a matter of considerable difficulty to observe their entrance into the vacuoles; nevertheless, figure 2 seems to illustrate such a condition, some of the mitochondria entering, and others apparently approaching, the cell vacuole.

Assuming the conception of these bodies as constituting the cell enzymes to be correct, we seem here to have before us a phenomenon identical with that observed in pancreatic cells. In the pancreas digestion is extracellular, and the mitochondria are extruded as zymogen grains into the pancreatic tubules; in Paramoecium digestion is intracellular, and the mitochondria are extruded consequently into the food vacuoles. Since, moreover, there is no evidence that the enzymes are extensively used up during their activity, it would seem to follow that in Paramoecia, excretion of enzymes must occur from the Paramoecium into the surrounding culture fluid whenever the contents of the vacuoles are expelled to the exterior.

SUMMARY.

1. Mitochondria are not artificial products, due to the action of fixatives on the protoplasm, for (a) they may be seen in living cells, (b) they may be stained intra-vitam with Janus green, (c) they may be separated from the remainder of the cytoplasm by centrifuging.

2. During the growth of the Paramoecium, the rod-shaped mitochondria increase by undergoing transverse binary fission; they undergo such fission irregularly, and apparently independently of the occurrence of genetic phenomena during the life cycle. At cyst formation they round themselves off. There is no evidence available to show that they arise *de novo* from the cytoplasm.

3. Mitochondria are extruded from the cell protoplasm into the food vacuoles. Bearing in mind their probable enzymatic nature, it is concluded that this

represents a process of intracellular digestion, similar to the process of extracellular digestion that occurs in gland cells, the secreted enzymes similarly arising in gland cells from the filamentous mitochondria.

I wish to take this opportunity of thanking Dr. O. W. Tiegs, of Melbourne University, for his valuable help and suggestions.

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THE FUNCTION OF THE LIPOID IN MITOCHONDRIA

by

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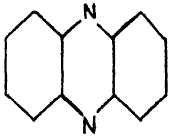
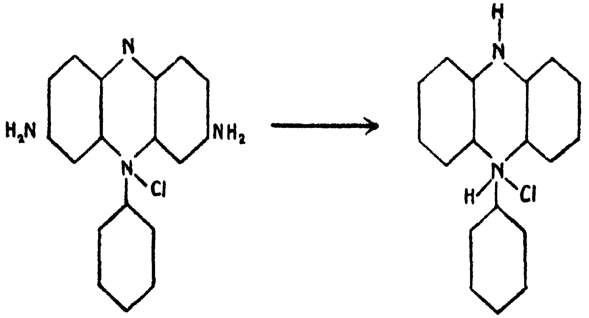
It has been shown by H. R. Marston (7) that the proteolytic enzymes form sparingly soluble compounds with dyes of the azine series, that is, with those dyes which contain the group:



The proteolytic substances are precipitated completely from crude enzyme solutions by the addition of soluble azines at suitable hydrogen ion concentrations. The point of union of the enzyme and the dye is indicated sufficiently clearly by the fact that the reduced "leuco" derivatives are no longer able to precipitate these enzymes:



Dye (red). Reduced leuco compound.

It is therefore the basic nitrogen of the azine nucleus which is responsible for the precipitation.

It is well known that certain azine dyes, such as Janus green, are specific staining agents for mitochondria. It is, in fact, through the employment of these dyes that the universal distribution of mitochondria in living, nucleated cells has been established (2). It has been suggested by Marston that the specific staining of mitochondria with azine dyestuffs may indicate that the intracellular proteolytic enzymes are concentrated in these bodies. "The mitochondria may thus be the site of syntheses in the cell, the water-poor phases which exist at the surface of the lipoid constituents of the mitochondria instituting favourable conditions for the synthetic activities of the enzyme."

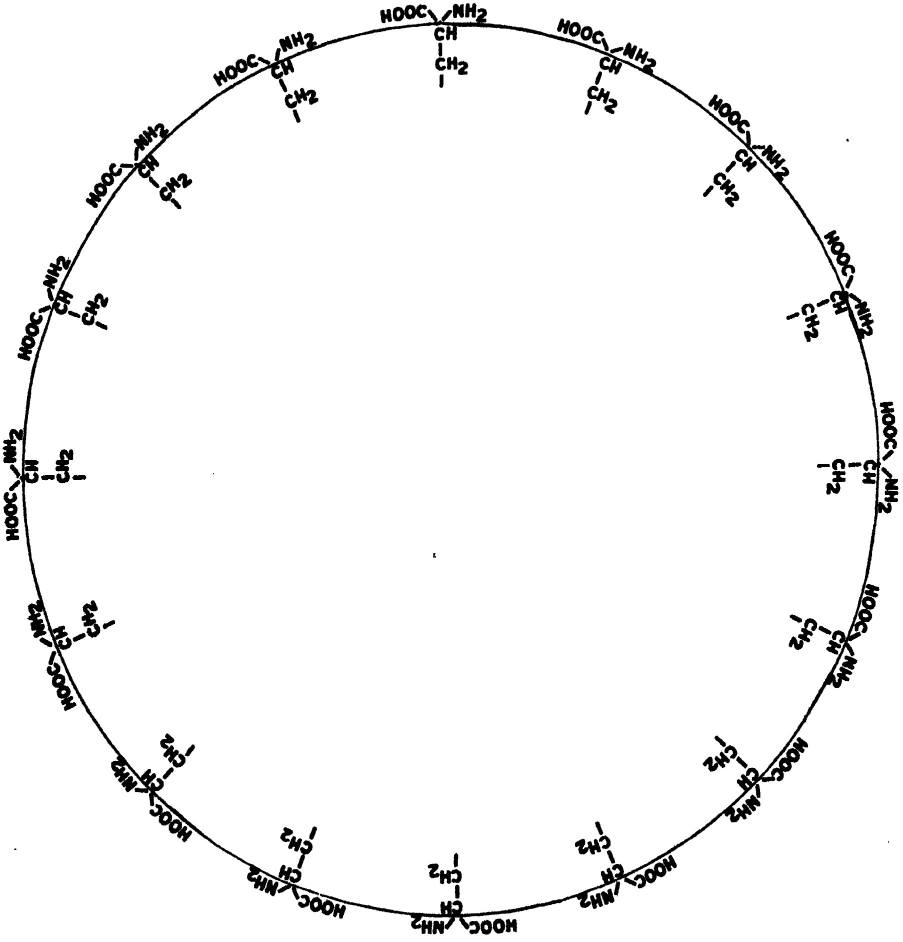


Figure 1. Illustrating the orientation of amino acids at the surfaces of mitochondria.

The lipoidal character of mitochondria is indicated by their solubility in alcohol, ether, chloroform, and other lipoid solvents, and by the fact that they frequently blacken when treated with osmic acid. Concentration of amino acids or other soluble products of digestion at the surface of a non-aqueous phase, in conjunction with synthesizing enzymes, would in itself afford a favourable condition for their re-synthesis into the colloidal constituents of the cell, but it is probable that in the synthesis of proteins, and possibly also of fats and polysaccharides, the lipoid of the mitochondria facilitates synthesis in another way as well, namely, by so orienting the molecules of the hydrolysate as to accomplish a concentration of reactive groups which would not otherwise be attainable.

The amino acids, with the exception of the heterocyclic acids, histidine, proline, oxyproline, and tryptophane, and the diamino acids, arginine and lysine, consist of the active groups, —COOH and —NH_2, attached to hydrocarbon chains of varying length. From the work of Langmuir on the orientation of fatty acid molecules at the surface of aqueous phases (6), and that of Harkins upon the orientation of molecules containing other active groups, such as the amino group itself (4), it appears that at the surface of a lipoidal phase the —COOH and – NH_2 groups of amino acids must be oriented towards the aqueous phase, while the lyotrope hydrocarbon radicles will be oriented towards and, probably, immersed within the lipoidal phase. Through the imposition of this orientation, which is diagrammatically illustrated in the accompanying figure, the movements of the molecules of hydrolysate must be greatly constrained, being confined to rotation around the axis constituted by the lyotrope carbon chain. Under any other conditions, even in the solid (non-crystalline) condition, the molecules must be oriented in every possible direction, and the mean distance apart of the reactive groups must be greater because every molecule may successively present any part of itself to the adjacent molecules. With all of the reactive groups oriented in one direction, and molecular motion constrained to rotation around the lyotrope axis, however, every molecule must present to adjacent molecules the one reactive group or the other, and the effective concentration of —COOH and —NH_2 groups must be immensely greater than that attainable by the mere removal of water from the reacting system.

It has been shown by Taylor (9), and the author (8), and latterly confirmed by Wasteneys and Borsook (11) that proteolytic enzymes may accomplish resynthesis of protein from amino acids, provided the enzyme is present in relatively high concentration. Concentration of the hydrolysate and elevation of temperature are favouring conditions for synthesis, but no concentration of the hydrolysate suffices to achieve complete resynthesis of amino acids into protein. The same has also been shown to be true in the synthesis of fats by lipase (1). So far as proteins are concerned, however, the low concentration of free amino groups in living tissues, and the high relative concentration of proteins, and their stability, unite to indicate that notwithstanding the high proportion of water contained in living cells, synthesis of amino acids into protein is virtually complete.

It appears probable that this relative completeness of synthesis is brought about through the uniform orientation and restraint of motion of the molecules of hydrolysate by the lipoidal constituents of the mitochondria. If this be so, then the same mechanism affords an explanation of a fact which otherwise appears to be at present inexplicable, the fact, namely, that all of the amino acids in protein are α-amino acids[1], that is, the amino group stands in the closest possible proximity to the carboxyl group. We can readily understand how this configuration must conduce to the stability of the resultant protein by shortening the chains which unite the amino acids to one another, but this fact does not explain how it is that the protein manufactured by living cells comes to be of this stable type. The mechanism of synthesis which has been suggested, however, would render it inevitable that the amino acids which unite to form proteins must be α-amino acids, because only acids of this configuration could simultaneously expose both of their active groups to the surface of the aqueous phase which contains the enzyme.

The single apparent exception to the rule that amino acids derivable from proteins are α-amino acids is that afforded by lysine, and the behaviour of this amino acid, when it enters into combination with others to form protein, affords the most remarkable confirmation that could be desired of the above interpretation of the mechanism of protein synthesis within the cell. For although lysine possesses both an α- and ω-amino group, and the latter, owing to its distance from the carboxyl, is probably the more basic group[2] (5), yet in proteins it is the α-amino group which is combined with the carboxyls of other amino acids, while the w-amino group remains uncombined, and actually constitutes the only free amino group in the whole of the protein molecule (10). Evidently in the synthesis of a protein the α-amino group of lysine must be brought into close proximity to the carboxyls of other amino acids, while the ω-amino group must be prevented in some fashion from attaining proximity to the carboxyl group of any other acid. It is difficult to see how this could be achieved by any mechanism other than one which maintains such an orientation of the amino acid molecules during the process of synthesis, that the carboxyls and adjacent amino groups are contiguous to, and other portions of the molecules removed from the region of chemical interaction.

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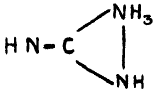
[1] Strictly, proline and oxyproline are not amino acids at all, but α-amino acids.

[2] On the other hand the slowness with which nitrous acid reacts with the ω-amino group of lysine (10) may indicate that this is the less active group of the two. Even if this be so, however, the weaker of the two amino groups in lysine is of about the same strength as the α-amino groups in aspartic and glutamic acids (5), and these are completely neutralized by carboxyl groups in proteins.

The inquiry may be raised why the ω-amino group of lysine, being an active group, does not also become oriented towards the water surface? It is not without significance in this connection that lysine contains the longest lyotrope carbon chain of any of the amino acids occurring in protein. We have at the one extremity of the chain two active hydrophil groups, at the other only one. Presumably the combined effects of the carboxyl and α-amino groups overbalance that of the single ω-amino group, and, because the chain is so long, oscillations of position would be difficult, the entire lyotrope effect of the intermediate chain of hydrocarbon groups having to be overcome before an inversion of orientation can be achieved. Similarly, in para derivatives of benzene, when the two groups in the para position are equally active the molecule lies upon the surface of water because both active groups cannot be simultaneously immersed, but if one group surpasses the other in activity it is drawn into the water, and the benzene ring is consequently tilted up on edge (4).

Arginine, as the structure of that molecule is ordinarily written, would appear to constitute another exception to the rule, but in fact it does not, for the work of Werner indicates that the guanidine derivatives, analogously to urea, are actually ring compounds (12). The structure of guanidine itself is probably:



so that if arginine is analogously built up it can only contain one amino group, namely, that which is in the α position. The absence in protein of any free amino groups corresponding to their arginine content is exactly what we should expect if the structure of arginine is analogous to that of guanidine and urea.

The α-amino groups in lysine are neutralized in proteins by carboxyl groups, and so are the α-amino groups in all of the other amino acids which participate in the construction of the protein. Just as many carboxyl groups must have been neutralized as amino groups, and since each amino acid has contributed at least one amino group for neutralization, each amino acid must also have contributed one carboxyl group. Only those carboxyls can remain free in the protein molecule, therefore, which are contributed by the second carboxyls of the dicarboxylic acids, glutamic acid, hydroxyglutamic acid, and aspartic acid. Proceeding upon the analogy afforded by lysine, we would infer that the free carboxyls must be those most distant from the amino groups, but unfortunately we lack as yet any means of distinguishing between the two carboxyls of a dicarboxylic acid in such a manner as to enable us to determine which of them remains unattached in the protein molecule.

So far as the amino acids derived from the aliphatic series are concerned, the theory herein presented is consistent with all of the known facts. It must be admitted, however, that data are as yet lacking which would enable us to predict the orientation at a lipoid-water surface of such molecules as those of histidine, tryptophane, and arginine. The fact that these also enter into the protein molecule through the agency of their α-amino groups, however, suggests that the combined activities of the carboxyl and α-amino groups overcome any affinity for the water phase which may be possessed by the cyclic portions of these molecules.

SUMMARY.

Following the suggestion of H. R. Marston, that the surfaces of the mitochondria are the loci of syntheses in the cell, it is pointed out that the lipoid composition of these bodies would tend to orient molecules at their surfaces in such a manner that the reactive groups, for example, the carboxyl and α-amino groups of amino acids, would all point towards the aqueous phase, while the lyotrope hydrocarbon chains would be buried in the lipoid phase. The movements of the molecules would thus be restrained and confined to rotation around the axis of the hydrocarbon chain, so that the reactive groups would attain an effective concentration, at the surface of the aqueous phase, which could not possibly be attained in any other manner. It is suggested that this affords an explanation of the completeness of enzymatic syntheses in living tissues, which has not hitherto proved attainable *in vitro* by mere concentration of the hydrolysate through removal of water from the reacting system. It is also pointed out that this mechanism of synthesis would necessitate the observed predominance of α-amino acids among the products of the hydrolysis of proteins, and would also render explicable the fact that lysine is united to the remainder of the protein molecule by its α-amino group, while the ω-amino group remains uncombined.

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THE PERMEABILITY OF POLARIZED MEMBRANES IN RELATION TO THE PERMEABILITY OF THE NUCLEUS AND THE PROPORTIONS OF THE VARIOUS AMINO-ACIDS WHICH ARE CONTAINED IN PROTEINS

by

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(Submitted for publication 12th May, 1926.)

It has been shown by Macallum (15) that the nucleus of the living cell lacks certain inorganic constituents, chlorides, phosphates, and potassium, which are characteristic components of the surrounding cytoplasm. It appears extremely probable from the evidence which is at present available that inorganic constituents, in the uncombined condition at all events, are totally absent from the nucleus, for while certain inorganic substances, such as iron for example, and possibly manganese, appear to be present in nuclei, it is possible that they exist therein in combination with organic radicles, just as phosphoric acid is present in combination with carbohydrate and nitrogenous bases to form nucleic acid. At all events, the absence of chlorides speaks very strongly against any interchange of inorganic salts between nucleus and cytoplasm, and we must infer that the nuclear membrane is of such a nature as to be impermeable to the inorganic electrolytes which are present in the cytoplasm which surrounds it.

This being the case, the nucleus must either be subjected to and capable of withstanding the very considerable osmotic pressure (about 7 atmospheres) of the inorganic electrolytes contained in cytoplasm, or else some other dissolved substances must take the place of these and maintain an osmotic pressure within the nucleus which approximates to the osmotic pressure of the surrounding cytoplasm. On a similar principle to that involved in the Donnan equilibrium we would expect to find such substances relatively concentrated within the nucleus, just as the inorganic electrolytes are relatively concentrated without.

It has been shown by Van Slyke and Meyer (24) that following upon absorption of the products of protein hydrolysis from the intestine, the amino nitrogen content of tissues increases up to a saturation limit which is characteristic for each tissue, and that thereafter the tissues become desaturated at varying rates. There is, therefore, a storehouse for amino acids within the cells, which is of somewhat variable capacity.

The possibility occurred to Collip (2) that the nucleus might form such a storehouse, the amino acids which it contains assisting to maintain osmotic equilibrium between its interior and its exterior. Accordingly he investigated the content of free amino groups in nucleated and non-nucleated cells which are as nearly as possible similar in other respects, the nucleated red corpuscles of birds, namely, and the non-nucleated corpuscles of the mammalia. He found the content of free amino groups far higher in the former, indicating the presence of far greater amounts of amino acids in nucleated cells than in non-nucleated cells of similar type and function. He concluded that the nucleus, among other functions, probably performs that of storing amino acids.

It must be admitted that this evidence, taken by itself, is not conclusive, for nucleated corpuscles differ from non-nucleated corpuscles in other particulars besides the possession of a nucleus. For example, nucleated erythrocytes contain mitochondria, while non-nucleated erythrocytes do not (3). Nevertheless, the properties and functions of the mitochondria, so far as we know or surmise them, do not suggest that they officiate as storehouses of amino acids, but rather as a means of converting them, when necessary, into protein (16). The mitochondria are composed very largely of lipoid materials, and amino acids are not lipoid-soluble substances (9). Amino acids could only be expected to accumulate at their surfaces, therefore, and in case they did so they would probably be converted into protein (22). There must, in fact, exist some method of preventing the access of excess of amino acids to the mitochondria, otherwise, it would appear, synthesis of protein would be the immediate response to ingestion of amino acids from the alimentary canal. This implies a means of storing the surplus amino acids in some other locality within the cell.

If, however, we accept the decision of Collip that the nucleus forms the storehouse of amino acids within the cell,[1] then certain curious implications are involved concerning the permeability of the nuclear membrane.

We have here a membrane which, while impermeable to ordinary electrolytes, its nevertheless permeable to amino acids, which are amphoteric electrolytes. The mere fact that it is penetrable by these substances would ensure their accumulation within the nucleus, because osmotic equilibrium is thereby maintained, but however great their relative concentration within the nucleus, this would nevertheless be determined by the lower concentration of amino acids in the cytoplasm, so that diminution of the latter would lead to issuance of amino acids from the nucleus to replace the deficit, and conversely augmentation of the amino acid concentration in cytoplasm would lead to a proportionate augmentation of amino acid concentration within the nucleus, the forces determining entry or exit being osmotic.

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[1] In certain unicellular forms (for example, Infusoria) the two functions of the nucleus, that of acting as storehouse for soluble food-reserves, and that of conveying hereditary qualities of the cell, are partially separated, and there are two nuclei, the micro- and the mega-nucleus, of which the latter has for long been generally supposed to officiate mainly as a storehouse of food-reserves.

What nature of membrane would permit the entry of amphoteric electrolytes, while interposing a barrier to the passage of electrolytes of a non-amphoteric character? The mere contrast of the permeabilities of these two classes of electrolytes suggests an electrical origin of the phenomenon, and, in fact, a membrane which is electrically polarized, might be expected to possess precisely the properties which Collip has inferred, are possessed by the nuclear membrane.

The effects of such a surface upon the orbits of approaching ions are illustrated in figure 1, in which the surface of the nucleus itself is represented as negatively charged, while that of the surrounding cytoplasm is represented as positively charged.[2] Any approaching positively charged ion would be repelled by the positive (induced) charge upon the cytoplasmic surface.[3] Any approaching negative charge would penetrate the positive layer but be repelled by the negative layer of charges. An amphoteric substance in solution, however, at any reaction in the neighbourhood of its isoelectric point, yields a proportion of doubly-charged, or "Zwitter-ions" (26). Such ions would be polarized by the electrical double layer itself, presenting their negative charges to the positively charged layer and their positive charges to the negatively charged layer. There would thus be nothing to prevent their penetration of or exit from a surface of this character. We may infer, therefore, that if Collip's evidence be taken as affording satisfactory proof that the nucleus acts as a storehouse for amino acids, then it must also be regarded as affording proof that the surface of the nucleus is electrically polarized.

The concentration of Zwitter ions in a solution of an amphoteric electrolyte rises rapidly as the reaction approaches the isoelectric point of the amino acid. From this it follows that the penetration of the nucleus by any particular amino acid will become much more difficult the further the P_H of the cytoplasm recedes from the isoelectric P_H of that amino acid. If all of the amino acids which gain entry into the circulating fluids and the tissues were presented to the surface of the nucleus in equal concentration, then that acid of which the isoelectric P_H approaches most closely to the P_H of the cytoplasm would accumulate more readily within the nucleus than any other. The concentration of any amino acid within the nucleus would in that event depend primarily upon the closeness of its isoelectric P_H to the actual P_H of the cell. The concentrations of acids having isoelectric points in this neighbourhood would be high; those having isoelectric points receding from this value on either side would be present in rapidly diminishing concentrations. The relative concentrations of amino acids stored within the nucleus, subsequently to become available, if required, for the synthesis of protein, would, therefore, in this event be proportional to some function of their Isoelectric P_H values, or:

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[2] The surface of the nucleus has a high oxidative potential (20, 25), and hence must contain an excess of negative charges. The retention of amino acids within the nucleus may thus depend upon the maintenance and magnitude of the oxidative potential at its surface.

[3] Or, which comes to the same thing, would take the place of one of the positive ions, which would, in turn, be repelled.

$$c = F(I. P_H),$$

where c is the concentration of any particular amino acid and $F(I. P_H)$ is some undefined function of the Isoelectric P_H, $(I. P_H)$.

Actually, however, amino acids are not presented to the cell in equal, but, on the contrary, in very unequal proportions, and since the forces which necessitate the passage of amino acids across the polarized membrane at the surface of the nucleus are osmotic forces, the concentration in which they are presented to the cell or to its nucleus must participate in determining the concentration which is attained within the nucleus itself. In other terms:

$$c = C\ F(I. P_H),$$

where C is the concentration in which the particular amino acid is presented to the surface of the nucleus. From this it follows that:

$$\frac{c}{C} = F(I. P_H),$$

that is, the ratio of the concentration of the amino acids *within* the nucleus to their concentration *without* the nucleus should be some undetermined function of the isoelectric P_H values for the acids, and this ratio should exhibit a sharp peak in the neighbourhood of the P_H of the cytoplasm, which possibly differs slightly in the cells of different tissues.[4]

We are not in a position to evaluate directly either of the quantities c or C. But if the proportion in which the various amino acids are stored within the cell represents the proportion in which they are available for the synthesis of protein by that cell, and they are totally utilized, then the amino acid composition of the hydrolysate derived from any protein should reflect fairly closely the relative proportions of amino acids stored within the nuclei of the cells from which it was derived. This would be true if the available acids were totally utilized and if the mitochondria played no part in *selecting* the amino acids which are to be reconstituted into protein, but passively accept whatever is presented to them.[5] It is possible that some selection does actually occur, since Harkins has shown that the proportions of various molecules which concentrate at a fluid

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[4] The observations of Harvey (7), Jacobs (11), and others demonstrate that the P_H of cell contents may not be directly inferred from that of the surrounding medium. It is usually more acid owing to accumulation of CO_2 within the cell (11).

[5] Cf. preceding paper.

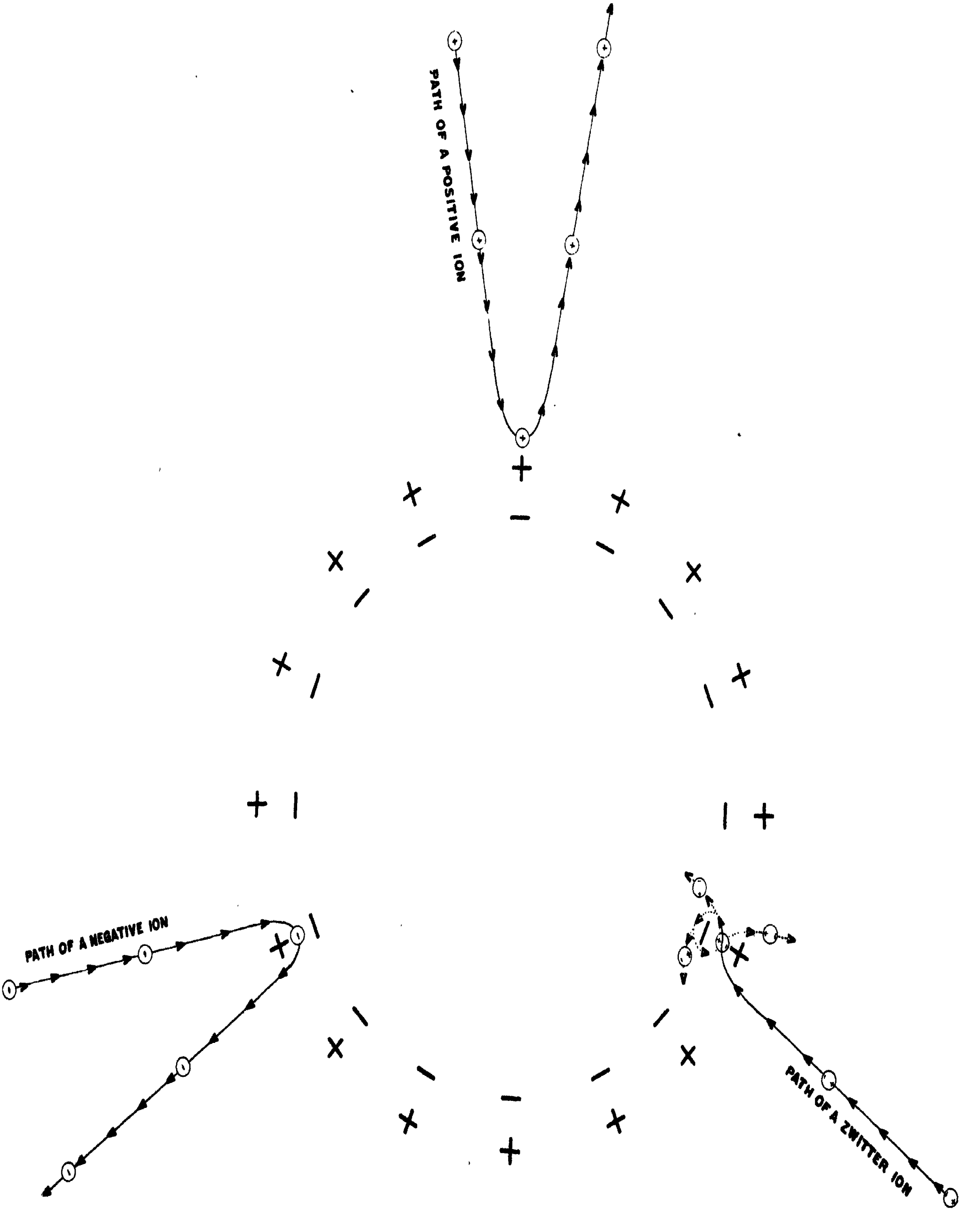

Figure 1. Illustrating the effect of polarization upon the penetrability of a surface by ions.

surface are determined, not solely by their relative concentration in the body of the solution, and the reduction of surface energy which they severally bring about, but also by the conformations of the molecules and the ease with which they can be fitted together to form a monomolecular layer (5). Still, admitting the probability that these factors may play an important part in determining the amino acid composition of the proteins, we may still affirm that the *availability* of the several amino acids for synthesis into protein must be a factor in deciding the proportions of amino acids which will enter into that protein, and since the concentration of Zwitter ions in an ampholyte solution presents such a steep maximum in the neighbourhood of the isoelectric point,[6] we would confidently expect to see this peak reproduced in the composition of a protein if Collip's hypothesis of amino acid storage were correct.

The values of C are still more difficult to ascertain. We cannot take them to be proportional to the concentration of amino acids in the dietary, for we know from the results obtained by Abderhalden, London, and Oppler (1) that amino acids are not absorbed from the alimentary canal in the proportions in which they are presented to the animal in the diet. The more closely the dietary proteins approximate to the composition of the body proteins, the more completely are they absorbed, and the less they correspond to the composition of the body proteins of the animal the more resorting by the alimentary canal is required and the more wastage of unutilizable amino acids is involved. These excesses of amino acid are passed on unabsorbed, and in consequence the "biological value" of the protein falls (23). The relative concentrations of the amino acids in the blood are unknown, for at present the resources of our analytical technique are insufficient to ascertain them. From the above-cited results of Abderhalden, London, and Oppler, however, and our general familiarity with the economy of effort consequent upon adaptation which characterizes living organisms,[7] we may infer with some confidence that, selection having been performed by the alimentary canal, it is final, and that all that is passed on to the tissues is potentially utilizable for constructing protein. If this be the case, then the total amino acid composition of the organism should reflect the normal proportions of amino acids which are presented to it, some being utilized predominantly in one type of cell and to produce one type of protein, others in other types of cell to produce other types of proteins, the wastage in the construction of the whole animal being a negligible minimum.

The amino acid composition of the whole animal is unknown, but Osborne and Jones have determined the amino acid composition of the muscle tissue of the ox (19). This is not quite such a representative tissue as we could desire for our purpose, but still it is a mixture of many kinds of cells, and doubtless

[6] Similar to the maximum of flocculation.

[7] The biological equivalent of the principle of Le Chatalier.

represents a closer approximation to the composition of the body as a whole than to that of any particular protein which it contains.

Our knowledge of the amino acid composition of protein hydrolysates has recently become more accurate, owing to the discovery by Dakin of the method of extracting the mono-amino acids from the hydrolysate with butyl alcohol (4). The aggregate nitrogen of the amino acids thus determined amounts to over 90 per cent. of the total nitrogen in the protein, previous methods yielding aggregate values of only 60 or 70 per cent. Unfortunately, only three proteins have as yet been analyzed by this method, namely, gelatin (4), casein (4), and lactalbumin (12).

The analysis of muscle tissue by Osborne and Jones was made before this method had become available. Comparison of Dakin's analysis of gelatin with older analyses indicates that in Osborne and Jones' analysis alanine and some other amino acids may be deficient, while glutamic acid may be in excess. Still the analysis accounts for over 70 per cent. of the total nitrogen, and the errors, as we shall see, do not suffice to mask, although they doubtless modify, the effect for which we are seeking.

Dividing the concentrations of the amino acids found in the hydrolysate from any protein by the concentration of the corresponding acids in muscle tissue (regarded as a sample, admittedly defective, of the whole animal), this ratio plotted against the isoelectric P_H values of the amino acids concerned should, if Collip's hypothesis is correct, yield a curve presenting a peak at the P_H of the cell in which the protein was manufactured, and falling rapidly on either side of this peak.

The results of plotting these ratios for gelatin, casein, and lactalbumin against the isolectric P_H values of the respective amino acids are shown in the graphs illustrated in figures 2, 3, and 4. The estimates of isoelectric P_H which are employed in these graphs are enumerated in the accompanying table, together with the sources from which they are obtained:

TABLE OF ISOELECTRIC P_H VALUES FOR AMINO ACIDS.

(Calculated from the formula: $-2P_H = \log Ka + \log Kw - \log Kb$
where $\log Kw = -13{\cdot}90$ at 25°C.)

Amino Acid.	Isoelectric P_H.	Author.
Aspartic Acid	2·76	Levene and Simms (14)
Glumatic Acid	3.23	Harris (6)
Cystine	4·30	Merrill (18)
Phenylalanine	5·35	Kanitz (13)
Tyrosine	5·65	Hitchcock (8)
Valine	5·95	Harris (6)
Leucine	5·97	Harris (6)

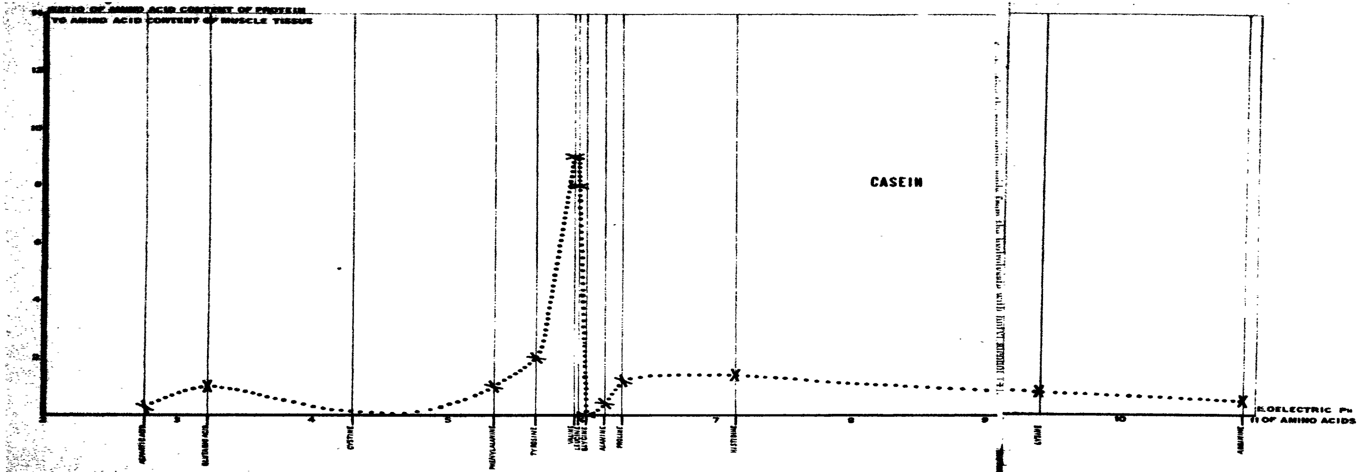

Figure 3. Showing the relationship between the proportions of various amino acids contained in casein and the isoelectric P_H values of these acids.

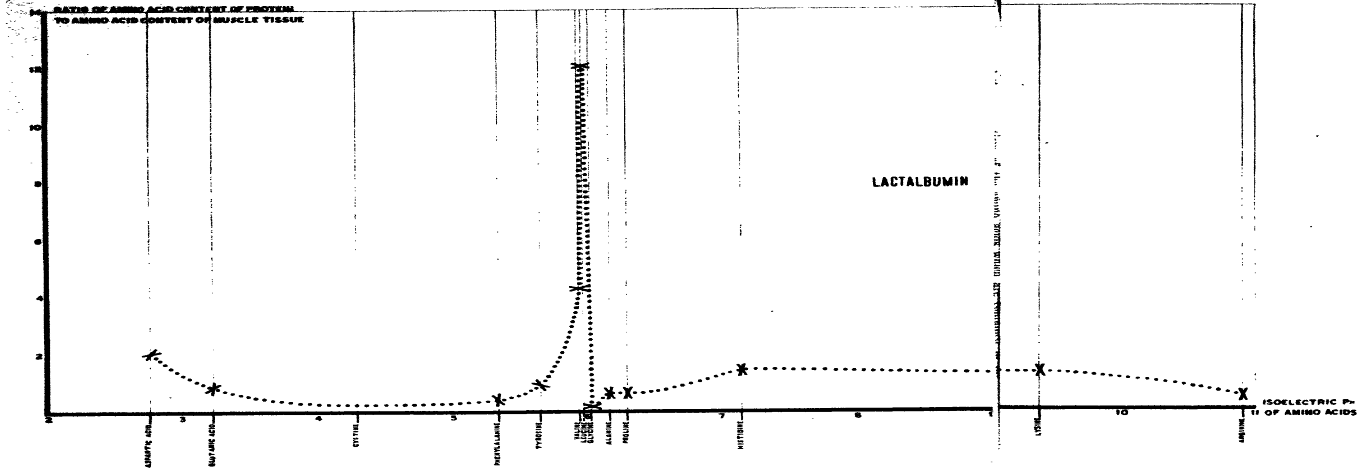

Figure 4. Showing the relationship between the proportions of various amino acids contained in lactalbumin and the isoelectric P_H values of these acids.

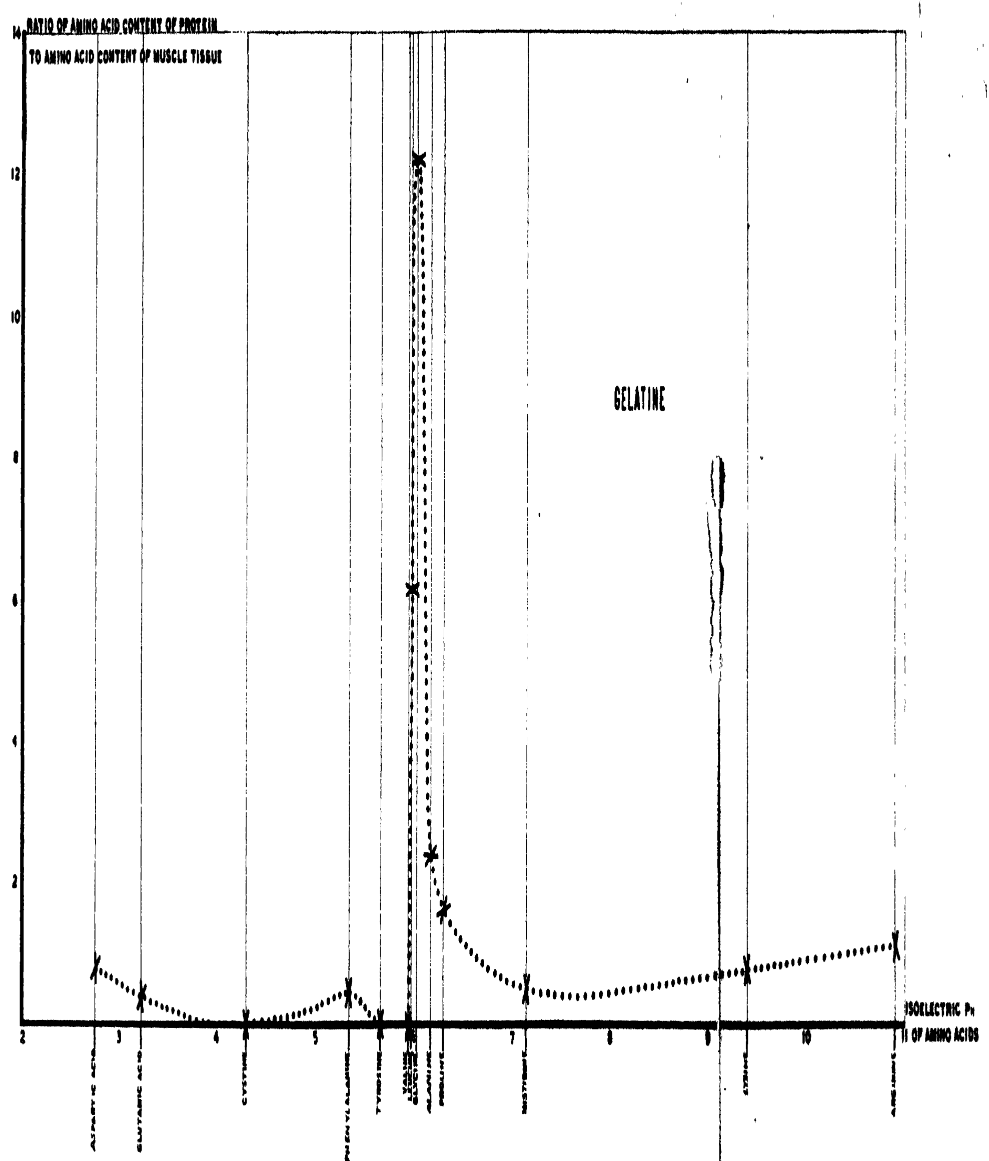

Figure 2. Showing the relationship between the proportions of various amino acids contained in gelatin and the isoelectric P_H values of these acids.

Amino Acid.	Isoelectric P_H.	Author.
Glycine	6·03	Harris (6)
Alanine	6·17	Harris (6)
Proline	5·30	McCay and Schmidt (17)
Histidine	7·15	Kanitz (13)
Lysine	9·40	Kanitz (13)
Arginine	10·97	Hunter and Borsook (10)

The results bear out our anticipations in rather a remarkable fashion, considering the number of uncertainties and approximations which are involved in obtaining them. The peak in each curve is so very prominent that the effect of polarization at the surface of the locus of amino acid storage is instantly suggested. Moreover, the peak is in each case in the neighbourhood of the P_H which we would expect in the contents of a living cell, a P_H, that is, a little on the acid side of neutrality (7, 11). The crowding of amino acid isolectric P_H values in this neighbourhood is also very significant, and suggests that in the synthesis of all proteins this factor plays an important rôle.

Other proteins have been plotted in a similar manner, and reveal similar sharp maxima in various regions, but when the uncertainties of the older analyses are superadded to the uncertainties of Osborne and Guest's analysis of muscle tissue, coupled with the poorly representative character of this tissue itself, the accumulation of errors may distort the curves so greatly that it has not been thought worth while to reproduce any others here. Some exhibit subsidiary maxima which may very easily have originated in the accumulation of analytical errors. In one case, however, we may be sure that the second maximum is real, namely, in the case of salmine, which contains 87 to 91 per cent. of arginine, no lysine or histidine, with 11 per cent of proline, and 4 to 5 per cent. of leucine. The circumstances under which the proteins in spermatozoa are synthesized, however, are so peculiar that one is not surprised to find their composition departing from the usual rule. Among other interfering factors we may mention the frequency of mitoses in spermatogenic tissue with dissolution of the nuclear membrane, and consequent scattering of amino acids through the cytoplasm, and temporary lapsing of selective storage.

The question may be raised whether our estimates of the isoelectric P_H values of amino acids, particularly those of glycine, valine, and leucine, are sufficiently accurate to assure us that they are distributed in the order represented in the graphs. Undoubtedly they are not. Thus if the isoelectric P_H value for leucine were *greater* than that for glycine, the lactalbumin curve would display *two* sharp contiguous maxima. If the isoelectric P_H value for leucine were *less* than that for valine, the curve for gelatin would display two maxima. Yet the estimated isoelectric P_H for leucine differs by only +0·02 from that for valine, and —0·06 from that for glycine. We are not entitled to suppose that these estimates have attained such a degree of accuracy as to ensure the correctness of such fine gradations of isoelectric values, and the single maxima displayed in these curves may therefore be accidental in origin.

The internal evidence afforded by the curves themselves, however, shows that the maxima are not accidental in origin. Apart from glycine, valine, and leucine, the ascertained values of the isoelectric P_H for the remaining acids are certainly of sufficient accuracy to guarantee that they are placed in their correct order upon the axis of abscissae. In each curve the presence of a maximum in the neighbourhood of $P_H = 6$ is indicated by several points upon the curve. Thus in the case of casein, which exhibits a maximum at valine, there are two points upon the descending limb of the curve upon the acid side (tyrosine and phenylalanine), and there is one point upon the more steeply descending limb on the alkaline side (glycine). In the case of lactalbumin, which exhibits a maximum at leucine, there are three points on the descending limb of the curve upon the acid side (valine, tyrosine, and phenylalanine), and these points descend in order of their distance from the maximum. In the case of gelatine, which exhibits a maximum at glycine, there is a point (leucine) which lies exactly upon the almost straight descending limb which intersects the axis at valine (absent in gelatin), and upon the less steeply descending limb of the curve on the alkaline side there are three points which descend in the order of their distance from the maximum, namely, alanine, proline, and histidine. The reality of the maxima thus indicated can hardly be doubted, and moreover the crowding of amino acids in the neighbourhood of $P_H = 6$, as pointed out above, contributes evidence from another angle pointing to the importance of isoelectric P_H in determining the facility with which amino acids can be employed in the manufacture of protein. The fact is probably not without significance, also, that of the three protein curves depicted in the graphs, those two (casein and lactalbumin) which are derived from the same organ (not necessarily the same cells) exhibit curves of like form, with a steeply descending limb on the alkaline side of the maximum, while gelatine, derived from a widely differing tissue, exhibits a curve which departs widely from this type, and displays a steeper descent on the acid than on the alkaline side of the maximum. Whatever uncertainty attaches to the absolute magnitudes of the isoelectric P_H values for the different amino acids, therefore, it appears highly probable that they are seriated in the correct order in the diagrams, and that the maxima in the curves are real, and point to the existence of a polarized surface within the cell which determines the availability of amino acids for synthesis into protein.

SUMMARY.

It has been shown by Collip that nucleated erythrocytes contain far greater amounts of free amino groups than non-nucleated erythrocytes. He infers that

amino acids, among other soluble substances, take the place of mineral constituents, which are absent from the nucleus, in maintaining osmotic equilibrium between the nucleus and the surrounding cytoplasm. It is pointed out that as the nuclear membrane is permeable to amphoteric electrolytes, but not to non-amphoteric electrolytes, it must be electrically polarized.

The relative proportions of the amino acids which are contained in the three proteins (gelatin, casein, and lactalbumin), which have been completely analyzed, point also to the existence of a polarized surface within the cell which determines the availability of amino acids for synthesis into protein.

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CORRECTION.

The following should occur as a footnote on p. 14 to

STUDIES ON THE REFRIGERATION OF MEAT, I.

by

G. A. COOK, E. F. J. LOVE, J. R. VICKERY AND W. J. YOUNG.

One of the authors (J.R.V.) held a Government Research Scholarship at the University of Melbourne, and the investigation was carried out under the direction of a sub-committee consisting of Professor Laby, Dr. Love, and Associate Professor Young, of that University.

ENDOWMENT OF THE JOURNAL BY SIR JOSEPH VERCO

The Board of Editors have much pleasure in announcing the munificent endowment of the Australian Journal of Experimental Biology and Medical Science, with the sum of £5,000, by Sir Joseph Verco. This sum will be held in trust by the Council of the University of Adelaide, and the income derived therefrom will be devoted to sustaining the Journal, which will become the property of the University.

The Editors wish to express their appreciation of the assistance which has thus been rendered by Sir Joseph Verco to the furtherance of research in the sciences fundamental to Medicine in Australia. The purpose and scope of the Journal will be the same as heretofore, and it will be conducted by the same editorial committee. As hitherto, the Journal will be available for the publication of any research of merit in its particular field which may be submitted to it.

The Editors furthermore desire to record their sense of the indebtedness of Medical Science and Education generally to the services and generosity of Sir Joseph Verco. Since the foundation of the Medical School of the University of Adelaide Sir Joseph Verco has been actively associated with it, and we are very largely indebted to him for the progress which it has achieved.

He was born at Fullarton, in South Australia, seventy-five years ago, and received his early education at the Adelaide Educational Institution, conducted by the late Mr. John L. Young, and afterwards at St. Peter's College, winning the Young Exhibition. He received his medical education at St. Bartholomew's Hospital School, and graduated a Bachelor of Medicine of the University of London in 1875.

His student career was marked with the greatest distinction. He acted as House Physician at St. Bartholomew's Hospital during 1876. He became a Doctor of Medicine in the same year, and a Fellow of the Royal College of Surgeons, England, in 1877. He then returned to Adelaide, where he was was engaged in an extensive and distinguished practice for many years. His learning and skill were at the disposal of the Medical School and the Adelaide Hospital. He was lecturer in Medicine at the University for twenty-seven years, and has acted as Honorary Physician and Consulting Physician at the Adelaide Hospital for forty-four years, and at the Adelaide Children's Hospital for forty-eight years. Throughout his busy life he has always shown the utmost interest in the welfare of scientific societies in South Australia. He was for nineteen years President of the Royal Society of South Australia, and is at present Honorary Curator of the Section of Mollusca of the South Australian Museum. He has filled all the positions of honour that could be bestowed upon him by his fellow-practitioners, as President of the South Australian Branch of the British Medical Association in 1886-1887, and for four years during the war, and was President of the first Intercolonal Medical Congress held in Adelaide in 1887.

Upon retiring from practice he gave most generously of his time to the Medical School as Dean of the Medical Faculty and member of the Council of the University for several years. He took a leading part in founding the School of Dentistry.

He resigned the Deanship of the Faculty of Medicine to undertake the direction of the newly-created Faculty of Dentistry, of which he is still Dean. As a member of the University Council and the Advisory Committee of the Adelaide Hospital, however, his services are still rendered generously available in the interests of the School of Medicine.

His contributions to medical literature include many communications to the medical publications of Australia, and he was associated with the late Sir Edward Charles Stirling in the authorship of the article on Hydatid Disease in Allbutt's "System of Medicine." He also contributed widely to the Proceedings of the Royal Society of South Australia.

His gift is a happy expression of his devotion alike to Medicine and to Science.

THE ACTION OF CARBONIC ACID ON HAEMOGLOBIN

by

WILLIAM ALEXANDER OSBORNE

(From the Physiological Laboratory, University of Melbourne).

(Submitted for publication 16th May, 1926.)

That haemoglobin is a salt compounded of a protein base, globin, and a complex organic acid, haematin, haemochromogen, or haem, is well known. The addition of acetic acid, as in the class exercise for making "acid haematin," produces globin acetate and liberates the complex haem, which like so many organic acids is soluble in ether, whilst the salts are insoluble. The object of the present investigation was to discover whether carbonic acid has a similar action, displacing the haem from its globin combination. The experiments were made with blood freshly drawn from the carotid artery of an anaesthetized dog.

If carbon dioxide gas, washed in aqueous sodium bicarbonate suspension, is bubbled through diluted blood the characteristic absorption band in the red of acid haematin will be detected on spectroscopic examination after several hours. That this is not methaemoglobin is shown by having a layer of ether above the blood, which may be undiluted, during the passage of the gas. This ether becomes eventually coloured, and gives the typical absorption band of haem in ethereal solution.

Three parts blood were added to 100 parts water supersaturated with CO_2. The mixture was shaken to get ride of excess CO_2, and sealed in a glass vessel suitable for spectroscopic examination. It was found that the absorption band in the red was not visible until five hours had elapsed. That this delay is due to the haemoglobin, and not to the qualities of the carbonic acid, is shown by adding blood to excess of an organic acid of approximately the same P_H. I have been unable to find in physico-chemical literature any figure for the P_H of water saturated at different temperatures with CO_2. The dissociation constant has been determined by more than one observer, but the results do not correspond. Taking the constant found by Kendall (1) for 18°C., namely, 3·12 x 10·7, and the known solubility in water at that temperature, the P_H should be 3·9. Dr. J. M. Lewis in my laboratory has found by the electric method, using a Leeds Northrup potentiometer and minimal contact wire electrodes, values for the P_H that lie close to 5. Blood was added to excess of acetic acid of P_{H_4}, and it was found that the slow change of colour and appearance of the spectrum of acid haematin were quite comparable with the action of carbon dioxide.

Dilute blood (3 in 100) was placed in a small rectangular bottle, and this was inserted in a Leonard Hill apparatus for studying the action of high gas pressures on small animals, in such a way that the spectrum could easily be seen through the thick glass windows. After subjection to 15 atmospheres CO_2 for twenty minutes no change was observable in colour or spectrum; longer exposures were unfortunately rendered impossible by a defect in the apparatus.

Blood diluted with water containing CO_2 at various tensions down to 10% atmosphere similarly showed the presence of haematin if sufficient time was allowed.

There is, however, a method by which the immediate formation of haematin can be detected, and that is to view highly diluted blood through a long tube. The dilution I have chosen is 1 in 3600, and the tube is 52 inches in length. The ends are closed with flat glasses from a polarimeter tube, and contact with metal is avoided on account of the ease of methaemoglobin formation. If the blood be freshly drawn from the carotid, and diluted with distilled water recently boiled, no band is seen in the red. But if the water contain a trace of CO_2 the band appears. Stale blood, twenty-four hours old and more, diluted with boiled water, gives a simlar band in the red, though the possibility of methaemoglobin formation must not be forgotten.

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ACUTE ENCEPHALITIS (X-DISEASE) AT BROKEN HILL

PROBABLE SUCCESSFUL TRANSMISSION TO SHEEP

by

J. LE M. KNEEBONE AND J. BURTON CLELAND

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(Submitted for publication 13th August, 1926.)

In January and February, 1925, cases diagnosed as acute encephalitis (X-disease) again appeared at Broken Hill. The symptoms and signs manifested corresponded with those already described in previous reports (1 to 6). The following points may again be emphasized: The considerable mortality, the high temperature often reached, the presence of coma, and often convulsions, the relative absence of eye lesions, the fairly rapid progress to death of the graver cases, and a general resemblance to cerebro-spinal fever, except that the cerebro-spinal fluid is clear instead of turbid. Increases in the leucocyte counts were also noted. We are of opinion that this symptomatology does not correspond with that of encephalitis lethargica. At the time these cases occurred there was no increase in ordinary cases of anterior poliomyelitis in the district, which increase would have been expected had these cases been encephalitic examples of this disease. An enquiry by one of us showed that only one case of undoubted ordinary infantile paralysis was reported in the district between November 1, 1924, and May 31, 1925. The apparent conveyance of the virus of this disease to sheep is also an important point of difference. We find in these further cases, and in the inoculation results evidence confirmatory of the view that the disease is a definite entity.

When several of these cases of encephalitis had appeared, one of us (J.K.) communicated with the other, and as a result it was determined to try to convey the virus of the disease by intracerebral inoculations to sheep. It will be remembered that in the first outbreaks of the disease in 1917 and 1918 the virus was apparently successfully conveyed to a series of sheep after its establishment in the monkey (1, 2, 6). We determined in this epidemic, if possible, to convey the disease directly from the human victim to sheep. Though the stress of work at Broken Hill was heavy at the time, inoculations were made into two sheep, as detailed later on, with what appeared to be successful results. A second series of inoculations were made from one of the first sheep, and a successful "take" in the third generation was also obtained, but not in the fourth. At this stage the press of other work, the difficulties entailed in obtaining animals and looking after them, and the expense, prevented a continuance of the experimental investigations. An effort was made to transplant the virus to Adelaide, but, unfortunately, the inoculations there were completely without effect.

It is realized that much more work might have been done in connection with the sheep successfuly inoculated; thus an examination of the brains of the animals that succumbed might have been much more extensive, with the object of showing still more clearly that no secondary bacterial organism was playing a part in the process. It was impossible, however, under the circumstances, to do much more than make the actual intracerebral inoculations, and after death ordinary blood-serum inoculations from the brains. It is realized that the failure to carry out all the checks possible detracts from the importance of the results. Nevertheless, we feel that what has been done should be put on record, and we hope that others who may have a chance to repeat these experiments under better conditions will avail themselves of any opportunity that arises.

Details of Inoculation Experiments.

Portions of the cerebrum, cerebellum, mid-brain, pons, medulla, and cervical cord of Lucy S. (Case 3), removed aseptically, were emulsified in a sterile mortar with 50% glycerine and water. The resultant product was used the same day to inoculate Sheep 1 and 2. In all cases the procedure used was the same, except that an anaesthetic was only used for Sheep 1 and 2. The hair was removed, the scalp thoroughly cleansed, and through a small incision a perforation was made through the skull in the parietal region (the perforation was made with a drill). It was found quite easy to tell when the skull was almost perforated, and a needle fixed to a 2 cc. syringe was used to inject 1½ cc. of the emulsion deeply and steadily into the brain substance. In most cases there was a little temporary unsteadiness or rigidity on one side as a result of this inoculation, although this cleared up rapidly, except in Sheep 1, in which it persisted for some days.

Sheep 1, inoculated on March 3, 1925, from the above emulsion, did not stand up for three days after recovery from the anaesthetic, and was very unsteady. It walked better the fifth day, and took food well for 24 hours, but then went off food again, became unsteady, and *died with general convulsions on March 11, 1925.*

Sheep 2 was inoculated on March 3, 1925. There was no noticeable change until the day before death, when a disinclination to feed was noticed. On the day of death, March 12, 1925, it kept wandering around in a circle, with the head retracted, and finally died after several general convulsions. (Both these animals were about a year old.)

Sheep 3 was inoculated with the emulsified brain of Sheep 2 on March 15, 1925, and died with similar symptoms to Sheep 2 on March 21, 1925.

Sheep 4 was inoculated with the emulsified brain of Sheep 3 on March 22, 1925. It kept apparently well for eight days, and died after convulsive attacks for 24 hours on March 31, 1925.

Sheep 5 was re-inoculated from the brain of Sheep 4 on April 1, 1925, but suffered no ill-effects. (It had been inoculated with the brain of Sheep 3 on March 15, 1925, without ill-effect.)

Sheep 3, 4, and 5 were lambs about three months old.

The brains from Sheep 1, 2, 3, and 4 were removed with strict aseptic precautions immediately after death, and preserved in 50% glycerine and water. They all showed the most intense pial congestion, with not the least evidence of suppuration in the needle track or elsewhere.

Complete bacteriological examinations were not carried out, but inoculations on to blood-serum from the needle track and surrounding brain tissue were made in each case with no growth resulting.

Microscopic Examination of the Nervous Tissue of the Sheep.

Sheep 1. Sections were made of the spinal cord, the medulla, pons, floor of the fourth ventricle, frontal and parietal regions. In all these perivenous cellular sheaths were present, sometimes to a marked degree. The cells present were lymphocytes, large mononucleated cells, and polymorpho-nuclears. No definite neuronophagocytosis was detected. No infiltration of the pia mater was present in the spinal cord, but there was some increase of the lymphocytes and a few polymorpho-nuclears in the meshes of the pia mater of the parietal region, and in some of the sulci perivenous sheaths were present. There were some cellular islands in the pons.

Sheep 2 showed similar lesions in sections of similar portions of the spinal cord and brain. There was marked sheathing of the vessels in places, such as in the medulla. Polymorpho-nuclears were fairly evident amongst the lymphocytes, etc. There were some islands of cells in the pons.

Sheep 3 yielded similar results. In the pia mater of the parietal region there was considerable infiltration with small cells, chiefly lymphocytes and large mononuclears.

In *Sheep 4* there had been a haemorrhage in the frontal region from the trauma of the injection. There remained some old blood-clot, surrounding which was an angiomatous-looking inflammatory reaction, consisting of large, irregular, thin-walled vessels, between which were swollen glial cells, some connective tissue cells, etc. Beyond the injured and reactionary area lymphoid perivenous sheaths were obvious. In the temporal region there were a few well-marked sheaths, but these were almost absent in the parietal area. No lesions were detected in the basal ganglia, cerebellum, or spinal cord, but in the pons were slight indications of perivenous sheaths.

DEATHS.

Case 1. Gladys K., aged 3 years, was admitted on January 17, 1925, in an unconscious condition, with a history of vomiting and drowsiness for some days; convulsions and delirium occurred 3 days previous to admission. The heart, lungs, and abdomen showed nothing abnormal. Head retraction and dyspnoea were present. The temperature was 103·2°F., the pulse 144, and respirations 44. Lumbar puncture yielded clear fluid under pressure. No organisms were detected in it by smears or culture. The child died without rallying.

Case 2. Mary P., aged 2 years and 6 months, was admitted on February 2, 1925, with a history of vomiting for two days and with convulsions on the day of admission. The bowels were quite regular. She had been well previously. On examination nothing abnormal was detected in the chest or abdomen. Head retraction was present, and Kernig's sign. Lumbar puncture yielded a clear fluid, with the pressure not much increased, and containing no organisms. The temperature was 104°F., the pulse 160, and respirations 34. Next day she seemed slightly easier. On February 4 she had a severe convulsion in the morning, and died, the temperature reaching 106·6°F. at the end. At the post-mortem examination the pial vessels were found to be very congested. There was no evidence of tuberculosis or suppurative meningitis. The mesenteric glands were enlarged. No other lesions were detected. Microscopical sections from four different parts of the brain showed the presence of definite islands of small cells, but perivenous sheaths were not detected. The small cells were sometimes shrunken, and occasional polymorphonuclears were amongst them. In one section, a small degenerated area, probably an infarct, was seen with shrunken nuclei and disappearance of the cerebral tissue.

Case 3. (From this case the sheep inoculations were made.) Lucy S., aged 2 years and 10 months, was admitted on March 1, 1925. She had had whooping-cough ten months before, but since then had been quite well up to two days ago. For the last two days she has had "turns" each day, lasting up to six minutes at a time, and three convulsions, followed by general twitchings during the day before admission. The bowels had been opened once daily. She had refused food since she became ill. Examination showed that she was in a semi-comatose condition, with generalized twitchings. Head retraction and Kernig's sign were present. Nothing abnormal was detected in the chest or abdomen. The urine showed no abnormality. The temperature was 101·6°F., the pulse 140, and respirations 48. Next day her condition was worse, although the twitchings were not so severe. There was left abducent paralysis. Lumbar puncture yielded 60 cc. of clear fluid under slightly increased pressure. This fluid contained no increase of globulin, no pus cells, and no organisms either in smears or on culture. The temperature, 101·6°F. on admission, rose in 12 hours to 105°F., and maintained a high level, rising to 106° a few hours before death, which occurred 36 hours after admission. At the post-mortem examination, 12 hours after death, the brain was removed with strict aseptic precautions. Very marked congestion of the vessels of the pia mater was present, both at the vertex and the base. There was no evidence of tuberculous or suppurative meningitis, and the other organs were healthy. Microscopic examination of sections from the brain and spinal cord failed to reveal the presence of perivenous sheaths of cells or of cellular islands. These sections included the basal nuclei. There was marked capillary congestion in the frontal and occipital areas.

Case 4. Guiseppe G., aged 26 years, was admitted on March 28, 1925. He was a miner, living in Broken Hill. There was a history of headache and loss of appetite five days before, but he had worked up to two days before admission. On the day before admission he was drowsy, and complained of headache. He became worse, hot, and delirious, and had vomiting and abdominal pain. Examination showed a well-built foreigner, who was very drowsy, and did not answer questions properly. The temperature was 105°F., the pulse 104, and respirations 28. The legs were held rigid, so that the knee-jerks could not be elicited. On March 30 he was still running a temperature up to 106°F. He could not be roused, but picked at the bed-clothes, and waved his right arm round. The leucocytes numbered 25,000 per c.mm. The Widal reaction was negative. By March 31 his condition had become gradually worse, but the pulse remained good up to the last, when death occurred from respiratory arrest. At the post-mortem examination the brain was very congested. The vessels of the pia mater were intensely engorged. Nothing abnormal of note was detected in the other organs, including the intestine. Microscopic sections of the frontal and occipital regions showed only congestion of the pial vessels. In the temporal region round one vein were a small number of lymphocytes, and near the surface a small island of degenerating small cells with pycnotic nuclei, some of the cells possibly polymorphonuclears. In the parietal area near a vein at the periphery there was a very small cellular island with irregular nuclei. No lesions were detected in the basal ganglia. In the pons there were a number of diffuse cellular islands scattered amongst the nerve cells, their nuclei irregular or flattened; in several places the brain tissue seemed fragmented or fibrillar, and scattered through it were irregular nucleated cells, some of them lymphocytes. In the medulla there were some scattered diffuse cellular islands with irregular nuclei.

Case 5. Allan R., aged 25 years, was admitted on April 26, 1925. He was a miner, living in Broken Hill. He had been quite well until four days before, when on returning from work he complained of severe pain over the right eye and slight abdominal pain. He vomited the same evening. He stayed in bed next day, with headache, abdominal pain, vomiting, and constipation. He became hot and feverish the same night, and could not sleep. The day before admission he had had a rigour, and became drowsy. On the morning of admission he became strange, stared about him, and would not speak. On examination he was drowsy, and did not answer questions. His temperature was 102°F., the pulse 132, and respirations 26. Nothing abnormal was detected in the heart, lungs, and abdomen. The white blood count was 13,750 per c.mm. On April 27 he could not be roused. There was plucking at the bed-clothes. In the afternoon the temperature rose to 106·2°F., the pulse to 152, and respirations to 32. On April 28 the temperature went up to 106·2°F. again. His general condition was worse. There was frequent spasmodic stiffening of the body, limbs, and neck. He gradually became more and more deeply unconscious, and died the same day. A blood-culture was negative. At the post-mortem examination all the vessels of the brain were intensely congested, but there was no exudate or other evidence of meningitis. The other organs were normal, including the intestine. Sections showed congestion of the vessels in the parietal and occipital regions, and in the medulla small cellular island and a tendency occasionally to an increase of cells in the perivenous spaces.

Case 6, Tibooburra Case (by kind permission of Dr. Keyes). J.S., a girl aged 8 years, was admitted at 3 p.m. on January 30, 1925, in a comatose condition, with a temperation of 102·4°F., pulse 112, and respirations 24. The mother stated that the child complained of not feeling well two days previously. As she was subject to "bilious attacks" she took little notice, and treated her for such (oil and restricted diet). The child vomited several times. She got out of her cot on the second night, which appears to have been the first symptom of cerebral involvement. Drowsiness came on then, and on the morning of admission the mother was frightened, which caused her to bring the child to the hospital. On examination the child was comatose, and definitely gave Kernig's sign. Heart and lung signs were absent; the urine was clear. The Babinski response was doubtful, and there is no record of subsequent Babinskis being positive. She was put on a mixture of hexamine 5 gr. 4-hourly. During the night the child was delirious and talkative. On the second day the temperature came down to normal, pulse 120, respirations 28. At 9 a.m. she had a convulsive attack. The left arm was stiff, the hand clenched, and jaws fixed. There was no opisthotonos. The attack lasted a few minutes. At 10 a.m. lumbar puncture was performed, which gave fluid under considerable pressure, but clear. From this time on spasms became more frequent, until by evening they came on every ten minutes. Opisthotonos increased progressively. At 9 p.m. the temperature was 106°F. The child was put into a cold pack, which reduced the temperature to 104·2°F.

Lumbar puncture was performed again in the evening, and the fluid obtained was under slight pressure. From then on the spasms were very severe until midnight, when they became less frequent and slightly less severe. There was extreme exhaustion. A 9 a.m. next morning the temperature was 104·2°F., pulse 140, respirations 36. Her condition seemed slightly better. The spasms ceased until midday, but during the afternoon she had about four spasms. Strophanthin 1/500 gr. was given at 11.45 p.m., and again at 3.30 a.m. Digitalis was administered at 1.45 a.m. by mouth. During this day she took slight nourishment, being able to swallow fairly well. During the night she had no spasm, but the pulse was almost uncountable. The temperature at 11 p.m. was 101·4° In the morning she lay in a stuporose condition with a temperature of 104·5°F. Strophanthin was administered at 10 a.m. Her condition became more and more stuporose, and she died at 1 p.m.

RECOVERIES.

Case 7. W.H.R., a male, aged 17, was admitted on February 22, 1925, in an unconscious state, from a station 240 miles away on the Queensland border. Four days previously he complained of pain in the back of the neck and between the shoulders, and was thought to be suffering from sunstroke. The day before admission he became unconscious, and was restrained with difficulty. He had had an attack of influenza two weeks before. Examination showed a thin-faced boy in an unconscious state. He would lie still for five to ten minutes, breathing through the mouth, and then stiffen the body and legs. There was twitching of the left side of the face. Temperature 102°F., pulse 96 (varying considerably), respirations 28 and of the Cheyne-Stokes type when quiet. He kept his eyes shut, -and resented having them opened. The pupils were small, equal, and reacted to light. There was nothing abnormal detected in the chest and abdomen. The urine contained a faint cloud of albumen, but no pus or organisms. By lumbar puncture 10 cc. of clear fluid were withdrawn under considerable pressure, which contained an increased number of cells (lmphocytes predominating), but no increase in globulin. There were no organisms present. The white blood cells numbered 17,500 per c.mm. The Wassermann test was negative. On February 23 he was still quite unconscious. On Febrary 25 he was conscious, but drowsy and listless, with the temperature normal. On March 1 he was much improved, with no headache, and talked rationally. On March 8 he was up, but his mentality seemed below normal.

Case 8. George G., aged 4½ years, was admitted on March 2, 1925. He had been quite well till the previous day, when he had headache and abdominal pain, followed by convulsions and vomiting. The bowels were normal, and there had been no dietetic errors. Examination showed a well-nourished boy with generalized twitchings. Head retraction and Kernig's sign were present. The heart, lungs, and abdomen showed nothing abnormal. By lumbar puncture clear fluid was withdrawn under slightly increased pressure, which showed an increased cellular content but no organisms. The temperature was 103·6°F., pulse 140, respiration 40.

Progress: The convulsions and rise of temperature only lasted for two days after admission. He then gradually improved, and was discharged cured on March 30, 1925. He vomited frequently during the acute stage of the illness, and had a screaming attack during convalescence. He was kept under the influence of sedatives, which seemed to have a marked influence on the convulsive phenomena.

Case 9. Alfred G. A., aged 4 months, was admitted on March 3, 1925. He was a perfectly healthy breast-fed baby until the previous day, when he seemed feverish and had twitching attacks. The bowels were quite normal. Examination showed a healthy-looking child with twitching of the face and hands. Kernig's sign and head retraction were present. He had a convulsion lasting three minutes shortly after admission. Lumbar puncture yielded clear fluid under slight increase of pressure. Nothing abnormal was found in the heart, lungs, and abdomen. Temperature 104·6°F., pulse 166, respirations 60.

Progress: There were twitchings with occasional convulsions until March 8, 1925, although the temperatures began to fall after two days. He was unable to swallow, and was fed with the stomach tube during this period. After March 9, 1925, he progressed well, and was discharged on March 29 apparently quite well.

Case 10. Jessie B., aged 2⁸⁄₁₂ years, was admitted on March 11, 1925. She had been well up to five days before admission, and then vomited and had a twitching attack. She was drowsy until three days previously, when she became very drowsy, with twitching of the face and arms. Two days before admission she became unconscious, and on the morning of admission the head had become bent back and the back arched. Examination showed marked head retraction, dyspnoea, colour poor, and Kernig's sign present. No paralysis was found or abnormality in other organs. Temperature 104·4°F., pulse 160, respirations 48. Lumbar puncture gave a clear fluid under slight pressure, with the globulin increased, and there was also increase in the cellular contents. There were no organisms in smear or culture. The urine was normal.

Progress: On March 13 the patient was slightly better, but would not take drinks. There was slight neck rigidity, with face and hand twitching. She had to be fed by stomach tube for fourteen days. The general condition gradually improved, and the temperature returned to normal, but the child had a vacant look, and took no notice of surrounding objects. Although there was no actual paralysis there was spasticity of the muscles of the neck and arm, and on her discharge on May 25, 1925, she could not walk properly. Some months later the child was able to walk and the mental condition had improved, but was still not quite normal.

SUMMARY.

1. Further cases of a form of acute encephalitis, occurring in the late summer in a dry, hot climate, are reported. The mortality was high.

2. The disease appears to have been conveyed to sheep by intracerebral inoculation from a human case, and transmitted to two other sheep in series.

3. Microscopic lesions in the human brains were slight, and in the case from which the sheep were inoculated only congestion has been detected.

4. As in previous reports, a sheep found refractory to a first inoculation has proved refractory to a second.

5. The results obtained support the view that this disease is a distinct entity, unless further and repeated attempts to convey the virus of ordinary acute polio-encephalitis to sheep eventually yield positive results.

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THE MILK OF AUSTRALIAN WOMEN

by

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The work described in this paper has been carried out to determine the variability of the milk secreted by normal women of Australian birth and European stock after lactation has reached a steady state. The connection between properties of the milk and certain factors relating to the mother and the child have also been examined. The variability during the earlier stages of lactation has been dealt with in a previous paper (Wardlaw, 16).

The women from whom the samples were taken were all adequately suckling their infants. The infant in each case appeared to be normal, and no history of any serious illness was obtained for it. In a few instances the mother's milk had been supplemented on certain occasions by other food, but in no case was the supplement habitual, nor had it been made use of during the period immediately preceding the examination.

A history was obtained from each woman, which included statements of her age, the number of children she had borne and suckled, the age and sex of the youngest child, the number of times it was suckled per day, and the nature of its motions. Information was also collected with regard to the prenatal condition of the mother, the nature of her last confinement, her occupation, diet, and other matters which are not dealt with in the present paper.

The infants were weighed naked to the nearest half-ounce at the institutions at which the samples of milk were collected.

The samples were obtained during the period between March and November, 1923. One sample only was taken from each woman. Except in one or two cases, in which a breast pump was used, the milk was obtained by massage of the breast. Care was taken to empty the breasts as completely as possible, not only to measure as accurately as possible the volume of milk obtainable in each case, but also on account of the variation of the fat content with the degree of emptying of the gland. It has long been known that the percentage of fat in the portion of the sample first withdrawn from the breast may be less than half that in the last portions (Söldner, 14). The similar behaviour of the concentration of fat in cow's milk during milking is also well known.

The concentrations of the other constituents of the milk are generally supposed not to be sensibly affected by the stage of emptying of the mammary gland. Talbot (15), however, has recorded a slight increase in the percentage of lactose as the breast is emptied.

Except in six cases, in which the samples were obtained from both breasts, the samples were obtained from one breast. The six samples obtained from two breasts are distinguished by asterisks in the table of results. The volumes in these cases have been halved to make them comparable with the figures for the other samples.

The subjects were instructed to feed their infants from one breast only at 6 a.m. on the day of collection of the sample, and to refrain from using either breast again until after the collection of the sample, which took place at about 10 a.m. Except where both breasts were emptied, the sample was obtained from the breast which had not been used last. The samples were therefore obtained after a minimum period of rest of four hours, although in most cases the period of rest must have been considerably greater than this.

The volumes of the samples obtained were measured to the nearest cubic centimeter. The analyses were commenced within about an hour of collection.

The specific gravity of the samples was determined by means of a pycnometer at a temperature of 25°C. The figures given are those compared with water at 25°C. The pycnometer used held about 2 cc. of milk.

The freezing point was determined with a Beckman thermometer by the method previously described by one of us (Wardlaw, 17).

The total solids were determined by weighing the solids obtained from the milk delivered from a calibrated 1 cc. pipette. The milk was dried for about six hours in a water oven.

Fat was determined on 5 cc. samples of milk from a calibrated pipette by the Röse-Gottlieb method.

Ash was determined on 5 cc. samples delivered from the same pipette as was used for the determinations of fat. The samples were dried in porcelain crucibles in a water oven, then ashed at a low red heat in a gas muffle. In cases where difficulty was experienced in getting rid of the last traces of carbon at the low temperature used, a little pure ammonium nitrate was added after cooling, and the heating repeated.

Lactose was determined by the Folin-McEllroy method (Folin and Denis, 5). One volume of milk diluted with nine volumes of water was used for the determination.

The percentages of protein were computed as follows: The difference between the percentage of total solids and the sum of the percentages of the individual constituents gives the percentage of protein and "extractives." From this the number 0·73 was subtracted as the average percentage of extractives.

The figure for the average percentage of extractives was obtained by subtracting the sum of the average percentages of lactose and ash for the present results (6·64%) from the average percentages of lactose, ash, and extractives (7·37%), obtained in a previous investigation (Wardlaw, 16), in which the protein was estimated directly by the method of Sikes (13) during the second week of lactation. Camerer and Söldner (1) have shown that this latter sum remains practically constant from this stage of lactation onwards.

RESULTS.

The results of the various estimations are recorded in the following table. Certain particulars with regard to the mother and child are also shown. The results are arranged in order of the age of the child or stage of lactation of the mother. Where several specimens of milk have been obtained at the same stage of lactation, they have been arranged in order of the volumes of the samples.

The figures in the different columns are as follows: (1) Serial number of sample; (2) Age of child in weeks to nearest half-week; (3) Age of mother in years to nearest year; (4) Weight of child at time of sampling the nearest tenth of a kilogram; (5) The number of times per day the child was suckled; (6) The volume of the sample to the nearest cubic centimetre; (7) The specific gravity at 25°C. compared with water at the same temperature; (8) The depression of freezing point; (9) Percentage of total solid matter; (10) percentage of fat; (11) Percentage of solids-not-fat; (12) Percentage of lactose expressed as anhydride; (13) Percentage of ash; (14) percentage of protein.

The table shows the results of examination of 120 specimens of human milk. The figure for the depression of freezing point is not available for some of the samples.

TABLE 1.

Composition and Properties of Human Milk from First Month of Lactation.

Number of Samples.	Age of Child, Weeks.	Age of Mother, Years.	Weight of Child, Kilograms.	Volume of Sample, c.cm.	Specific Gravity, 25°/25°.	Dep. of Freezing Point.	Total Solids, per cent.	Fat, per cent.	Solids, not Fat, per cent.	Lactose anhyd., per cent.	Ash, per cent.	Protein, per cent.
25	3	32	2.63	46	1.0269	—	13.35	4.8	8.55	5.13	0.21	2.52
16	4	26	3.3	28	1.0287	—	13.35	5.32	8.03	6.50	0.17	0.65
87	4	21	3.4	34	1.0283	0.592	14.56	5.45	9.11	6.26	0.24	1.92
133	4	33	4.2	54	1.0312	0.579	13.23	4.29	8.94	6.74	0.13	1.20
99	4	20	4.2	100	1.0307	0.573	13.86	5.04	8.82	6.45	0.16	1.51
131	4	39	4.9	100	1.0332	0.577	11.70	2.59	9.11	6.60	0.21	1.60
120	4.5	24	3.5	72	1.0312	0.590	14.25	4.47	9.76	6.20	0.26	2.61
122	4.5	32	5.0	76	1.0287	0.593	14.94	5.95	8.99	6.60	0.20	1.50
117*	4.5	36	4.6	50	1.0325	0.604	12.31	3.15	9.16	6.65	0.19	1.62

Number of Samples.	Age of Child, Weeks.	Age of Mother, Years.	Weight of Child, Kilograms.	Volume of Sample, c.cm.	Specific Gravity, 25°/25°.	Dep. of Freezing Point.	Total Solids, per cent.	Fat, per cent.	Solids, not Fat, per cent.	Lactose anhyd., per cent.	Ash, per cent.	Protein, per cent.
27	5	29	3.7	40	1.0274	—	15.64	6.61	9.03	6.35	0.12	1.87
20	5	40	3.6	42	1.0301	—	12.94	4.24	8.70	6.76	0.14	1.10
114	5	20	4.2	44	1.0316	0.595	13.02	3.98	9.04	6.70	0.19	1.45
123	5	26	4.5	55	1.0284	0.594	14.20	5.57	8.63	6.50	0.22	1.21
39	5	30	4.8	80	1.0322	0.582	11.61	2.65	8.96	6.45	0.22	1.60
134*	6	20	3.9	16	1.0317	0.581	13.28	4.31	8.97	6.82	0.09	1.36
80	6	28	4.7	36	1.0281	0.585	14.56	5.83	8.82	6.55	0.12	1.45
130	6	23	4.3	40	1.0310	0.580	13.25	3.99	9.26	5.73	0.21	2.63
11	6	24	3.7	50	1.0285	—	14.15	5.08	9.07	6.50	0.17	1.71
106	6	24	4.4	50	1.0302	0.586	14.02	5.39	8.63	6.50	0.18	1.10
115	6	21	4.3	66	1.0303	0.622	13.80	4.92	8.88	6.45	0.20	1.53
102	6.5	27	5.5	134	1.0303	0.582	14.14	5.34	8.80	6.26	0.14	1.71
12	7	24	4.2	38	1.0286	—	13.86	4.92	8.94	6.60	0.10	1.54
68	7	19	5.2	40	1.0285	0.578	15.46	6.25	9.21	6.50	0.23	1.78
9	7	22	2.9	40	1.0302	—	11.07	2.33	8.74	4.90	0.33	2.83
48	7	35	5.8	70	1.0274	0.574	14.99	6.39	8.60	6.40	0.21	1.28
90	7	28	4.5	70	1.0319	0.572	11.69	2.95	8.74	6.60	0.18	1.25
1	7	33	5.0	80	1.0305	—	13.00	4.12	8.88	7.20	0.13	0.84
88	7	32	5.4	90	1.0303	0.596	13.44	4.52	8.92	6.60	0.16	1.47
124	7	29	5.0	120	1.0317	0.596	13.81	4.21	9.60	6.55	0.21	2.15
104	7	23	5.1	140	1.0313	0.573	12.58	3.80	8.78	6.45	0.20	1.44
101	7.5	30	3.8	28	1.0316	0.577	15.32	5.91	9.41	6.07	0.13	2.53
94	7.5	27	4.9	38	1.0295	0.567	14.63	5.72	8.91	6.35	0.19	1.68
96	7.5	33	4.7	54	1.0337	0.582	12.72	3.44	8.28	6.30	0.19	1.09
6	8	32	3.4	20	1.0294	—	13.31	4.53	8.78	6.76	0.20	1.12
41*	8	29	4.8	10	1.0293	0.583	11.99	3.38	8.61	6.58	0.23	1.24
8	8	21	3.4	30	1.0293	—	12.70	4.08	8.62	6.60	0.23	1.09
34	8	39	4.9	46	1.0308	0.574	11.50	2.93	8.47	6.35	0.17	1.34
89	8	22	5.7	50	1.0300	0.586	14.32	5.16	9.16	6.76	0.20	1.50
116*	8	22	5.2	38	1.0278	0.605	17.00	7.72	9.28	6.55	0.17	1.83
125	8	20	4.9	86	1.0293	0.589	14.35	6.13	8.22	6.50	0.21	0.80
113	8	19	4.1	86	1.0297	0.584	14.14	5.18	8.96	6.50	0.25	1.51
105	8	29	5.6	86	1.0296	0.589	13.86	5.16	8.70	6.55	0.15	1.30
79	8	27	4.6	100	1.0296	0.591	13.08	4.24	8.84	6.35	0.13	1.66
110	8	23	5.5	110	1.0287	0.583	15.21	6.32	8.89	6.44	0.22	1.56
100	8	33	5.4	190	1.0312	0.602	12.53	3.50	9.03	6.40	0.18	1.75
30	8.5	24	4.3	36	1.0281	0.554	15.39	6.44	8.95	6.45	0.25	1.56
127	8.5	33	4.3	46	1.0316	0.590	14.42	5.77	8.65	6.40	0.18	1.33
97	9	23	4.5	60	1.0282	0.596	15.29	7.03	8.26	6.35	0.17	1.04
7	9	25	4.4	70	1.0286	—	12.59	4.22	8.37	5.79	0.22	1.66
49	9	23	5.6	100	1.0281	0.592	14.19	5.65	8.54	6.45	0.19	1.19
92	9.5	18	4.6	36	1.0305	0.558	11.65	3.22	8.43	6.55	0.16	1.01
95	9.5	30	5.9	48	1.0310	0.580	13.13	4.28	8.85	6.50	0.13	1.53
66	9.5	23	6.1	65	1.0306	0.582	16.06	7.25	8.81	6.50	0.26	1.34
128	9.5	30	4.7	66	1.0293	0.590	15.24	5.67	9.57	6.60	0.20	1.63
50	10	21	6.1	20	1.0238	0.569	18.51	10.29	8.22	6.35	0.22	0.94
62	10	20	4.3	56	1.0302	0.565	12.89	4.05	8.84	6.50	0.22	1.42
10	10	20	5.3	90	1.0273	—	13.41	4.97	8.44	6.20	0.17	1.36
81	10	22	5.8	90	1.0317	0.583	12.00	2.86	9.14	6.50	0.16	1.78
43	10	33	5.3	100	1.0289	0.579	13.60	5.01	8.60	6.50	0.14	1.25
67	10	20	5.8	157	1.0318	0.578	13.01	4.07	8.94	6.40	0.25	1.59
93	10.5	33	5.5	30	1.0257	0.568	17.35	9.18	8.17	6.21	0.20	1.06
112	11	21	5.8	66	1.0293	0.600	14.31	5.42	8.89	6.40	0.21	1.59
83	11	22	6.3	70	1.0302	0.600	13.14	4.10	9.04	6.40	0.33	1.52
137	11	33	6.9	94	1.0265	0.576	14.83	5.73	9.10	6.74	0.21	1.45
24	12	28	6.0	32	1.0284	—	11.65	3.45	8.20	6.20	0.16	1.13

Number of Samples.	Age of Child, Weeks.	Age of Mother, Years.	Weight of Child, Kilograms.	Volume of Sample, c.cm.	Specific Gravity, 25°/25°.	Dep. of Freezing Point.	Total Solids, per cent.	Fat, per cent.	Solids, not Fat, per cent.	Lactose anhyd., per cent.	Ash, per cent.	Protein, per cent.
56*	12	26	5.8	16	1.0267	0.550	14.78	6.40	8.38	6.40	0.15	1.12
109	12	30	5.1	52	1.0305	0.590	13.55	4.67	8.88	6.30	0.26	1.53
54	12	21	5.0	52	1.0295	0.573	14.41	5.40	9.01	6.70	0.17	1.43
51	12	27	5.9	54	1.0296	0.580	12.62	3.87	8.75	6.40	0.20	1.45
5	12	23	5.3	60	1.0276	—	15.20	6.28	8.92	6 87	0.18	1.16
23	12	34	5.7	90	1.0259	—	13.46	5.00	8.46	6.35	0.12	1.28
103*	12	22	5.8	46	1.0328	0.587	13.37	3.88	9.49	6.35	0.13	2.23
18	12.5	26	5.0	100	1.0308	—	13.14	4.08	9.06	6.55	0.17	1.65
129	13	27	6.9	44	1.0306	0.597	12.56	3.70	8.86	6.55	0.18	1.44
3	14	20	8.5	34	1.0306	—	14.28	5.18	9.10	6.80	0.17	1.44
28	14	40	5.3	40	1.0279	—	15.11	6.48	8.63	6.45	0.10	1.37
61	14	32	4.0	60	1.0278	0.584	15.33	6.27	9.06	6.45	0.27	1.63
78	14	29	4.8	70	1.0301	0.587	12.22	3.44	8.77	6.99	0.13	0.95
63	14	36	5.4	76	1.0329	0.585	12.28	2.39	9.89	6.82	0.27	2.10
36	14	21	5.8	80	1.0307	0.584	12.65	3.97	8.68	6.50	0.23	1.25
26	14	26	4.8	95	1.0318	—	12.60	3.39	9.21	6.50	0.08	1.95
64	14	24	6.7	168	1.0294	0.580	13.15	4.50	8.65	6.50	0.23	1.22
98	15	33	6.7	36	1.0287	0.586	13.75	4.45	9.30	6.45	0.10	2.06
2	15.5	24	5.5	26	1.0279	—	13.07	4.97	8.10	5.80	0.14	1.47
46	16	24	4.4	24	1.0272	0.556	14.64	5.99	8.66	6.45	0.11	1.43
59	16	25	6.6	30	1.0288	0.573	14.25	6.26	7.99	6.50	0.17	0.60
57	16	23	5.4	38	1.0275	0.580	14.44	5.77	8.67	6.45	0.13	1.39
44	16	40	6.0	44	1.0262	0.556	13.40	5.16	8.24	6.49	0.13	0.92
33	16	23	7.3	110	1.0278	0.553	13.63	5.21	8.42	6.45	0.18	1.09
76	17	31	6.1	54	1.0260	0.577	15.00	6.34	8.66	6.08	0.22	1.66
118	17.5	31	8.7	100	1.0309	0.579	12.45	3.65	8.80	6.50	0.16	1.44
86	18	35	6.1	33	1.0276	—	15.21	6.53	8.68	6.45	0.20	1.33
13	18	24	6.6	50	1.0299	—	12.34	3.50	8.84	6.65	0.13	1.36
52	18	30	6.3	59	1.0293	0.580	14.18	5.23	8.95	6.40	0.15	1.71
32	18	38	7.6	68	1.0308	0.565	12.39	3.53	8.86	6.45	0.20	1.51
15	18	29	7.0	70	1.0266	—	15.58	6.69	8.89	6.25	0.26	1.68
65	18	18	6.2	75	1.0247	0.585	14.05	4.68	9.37	6.45	0.20	2.03
75	19	29	8.1	64	1.0293	0.576	13.42	4.64	8.78	6.26	0.24	1.59
85	19	32	6.1	74	1.0283	0.586	14.51	5.75	8.76	6.55	0.21	1.30
73	20	32	6.8	52	1.0304	0.584	12.10	3.37	8.73	6.30	0.20	1.53
37	20	23	6.4	60	1.0271	0.582	14.93	5.84	9.09	6.45	0.21	1.74
60	20	29	5.9	95	1.0282	0.570	13.09	4.71	8.38	6.40	0.15	1.12
53	20	23	7.2	100	1.0323	0.570	11.33	2.55	8.78	6.60	0.18	1.30
14	21	22	7.3	48	1.0253	—	15.65	7.16	8.49	6.45	0.11	1.22
69	21	23	6.9	74	1.0290	0.584	14.30	5.46	8.84	6.50	0.21	1.43
107	21	37	7.4	100	1.0317	0.575	11.90	3.37	8.53	6.40	0.15	1.28
70	22	22	7.7	30	1.0258	0.545	15.87	7.68	8.19	6.40	0.18	0.90
108	22	24	7.5	32	1.0283	0.597	15.34	7.37	7.97	6.65	0.17	0.44
72	22	24	6.7	35	1.0247	0.562	16.82	8.68	8.14	6.40	0.27	0.77
71	22	26	6.7	80	1.0327	0.597	12.16	2.90	9.26	6.50	0.15	1.92
74	22	26	7.7	130	1.0309	0.592	12.89	3.98	8.91	6.35	0.14	1.72
82	24	22	6.3	36	1.0262	0.560	13.98	5.78	8.20	6.55	0.21	0.72
40	24	25	9.5	60	1.0305	0.562	12.39	3.72	8.67	6.14	0.21	1.62
4	26	28	7.6	80	1.0281	—	13.24	3.90	9.34	7.10	0.09	1.45
77	26	31	9.0	152	1.0288	0.586	13.76	5.25	8.51	6.26	0.18	1.37
55	27	20	7.6	36	1.0284	0.560	14.01	5.43	8.58	6.55	0.12	1.21
31	28	26	7.4	40	1.0271	0.551	15.75	7.60	8.15	6.71	0.20	0.53
29	28	21	8.6	68	1.0274	—	13.74	5.38	8.36	6.40	0.16	1.10
22	28	36	9.5	100	1.0332	—	11.23	2.56	8.67	6.93	0.16	0.87
47	34	43	11.1	124	1.0301	0.587	12.81	4.05	8.76	6.60	0.11	1.40

To summarize the information contained in the above figures the following table has been prepared (Table 2). In this table mean and extreme values are shown for each quantity, and also figures giving the dispersion or variability of each series of measurements.

Three types of mean value are shown: the arithmetic mean, the mode or value round which the results are most closely grouped, and the median or value above and below which equal numbers of the results occur. The upper and lower quartiles, or values between which 25% of the results on either side of the median are to be found, are given also. By dividing the difference between the two quartiles by twice the value of the median the figures given as dispersion have been obtained. By these figures the relative scattering of the different series of results about their median values can be compared.

TABLE 2.

Variability of Composition and Properties of Human Milk after first month of lactation.

				Quartiles				Dispersion.
	Mean.	Mode.	Median.	Upper.	Lower.	Max.	Min.	
Volume	66 cc.	40 cc.	58 cc.	84 cc.	40 cc.	168 cc.	10 cc.	0.379
Fat	4·95%	5·30%	4·85%	5·75%	3·86%	10·3%	2·3%	0.195
Ash	0·18%	0·19%	0·18%	0·21%	0·145%	0·33%	0·08%	0·188
Protein	1·41%	1·40%	1·40%	1·58%	1·15%	2·77%	0·41%	0·152
Specific gravity ..	1·0293	1·0296	1·0294	1·0307	1·0280	1·0337	1·0238	0·063
Total solids	13·74%	13·87%	13·67%	14·53%	12·82%	18·51%	11·07%	0·063
Solids-not-fat	8·79%	8·82%	8·82%	9·00%	8·59%	9·89%	7·97%	0·023
Lactose	6·46%	6·45%	6·49%	6·55%	6·36%	7·20%	4·90%	0·0146
Δ	0·580°C	0·583°C	0·582°C	0·589°C	0·573°C	0·622°C	0·545°C	0·0052
Calories, per 100 gm.	76·9	80·0	75·9					

The various quantities measured have been arranged in the above table in descending order of variability as expressed by the figures for the dispersions.

The most variable quantity is the volume of milk obtained from the breast, for which the dispersion is 0·379. Next come the variabilities of the percentages of fat, ash, and protein, for which the respective dispersions are 0·195, 0·188, and 0·152. These three constituents form a group, the variability of the concentration of each member of which is close to that of the others.

The variabilities of these quantities are about half that of the volume of the sample. The variability of the percentage of total solids and of the difference between the specific gravity of the samples and that of water comes next, with a dispersion of 0·063. The variability of this property is about one-third that of the preceding group. The variabilities of the percentages of solids-not-fat and of lactose follow with dispersions of 0·023 and 0·0146. The last figure is about one-tenth that of the percentages of the other constituents, fat, protein, and ash. Lactose is therefore by far the most constant or least variable of the constituents measured. The depression of the freezing point, for which the dispersion is 0·0052 has a variability only about one-third of that of the lactose. This quantity is thus the most constant of all those measured in the present investigation.

In the case of each of the measurements, except those of the volume of milk obtained from the breast, and to a less extent of the percentage of fat, the values of the arithmetic, mean, mode, and median lie close together in each series of results. There are considerable differences between the values of the arithmetic mean, mode, and median of the volume of sample for which the figures are respectively 65 cc., 58 cc., and 40 cc. The difference between the mean and the mode amounts to over 50% of the latter. These figures show that there is a preponderance of values above the mode over values below the mode.

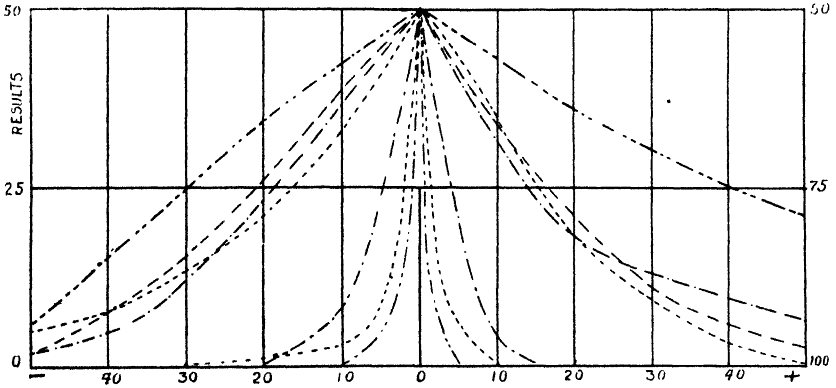

Figure 1.

Deviation from median %. Deviation from median %.

Variability of human milk: Abscissae: Percentage deviation from median. Ordinates: Percentage of total number of results. Legend (reading downwards): - - · —— - - volume of sample; —·—·- - percentage of fat; — · —— · percentage of ash; – – – – percentage of proteins; —·—·- · specific gravity; - - - - percentage of lactose; —·-·——·- - depression of freezing point.

The differences between the mean, mode, and median of the percentages of fat are much less, the figures being respectively 4·95, 5·30, and 4·85. The difference between the mean and the mode in this case amounts to less than 10% of the latter. Here the values greater than the mode only slightly outweigh those below the mode.

The values of the means, modes, and medians of the remaining quantities show no significant differences. These values are: For the percentage of ash, 0·184, 0·190, and 0·181; for the percentage of protein, 1·41, 1·40, and 1·40; for specific gravity, 1·0293, 1·0296, and 1·0294; for percentages of lactose, 6·46, 6·45, and 6·49; for depression of freezing point, 0·580, 0·583, and 0·582. These figures show that the distribution of values about the mode is approximately symmetrical, and that there is no preponderance of values on one side of the mode over those on the other side.

The values which show the greatest differences between their mean, mode, and median, that is, the volume of the sample and the percentage of fat, and also those which have the greatest variability. The variabilities of these values are not strictly comparable with those of the other quantities measured. The volume of milk obtained from the breast is dependent upon two factors: the volume of milk present in the mammary gland, and the proportion of this which is removed.

The degree of completeness with which the mammary gland is emptied will in turn affect the percentage of fat in the sample obtained. Any variation of the percentage of fat will also affect the specific gravity of the sample, and this property also will be affected by the manner of emptying of the gland.

The percentages of ash, protein, and lactose, on the other hand, and the depression of the freezing point are not, or only slightly, affected by the stage of emptying of the gland at which the sample is taken. These values are determined by factors within the maternal organism, and are not influenced by outside factors.

When the present figures are compared with those given by various authors for the milk of European and American women the ranges of variation of the different constituents are seen to show no significant differences. In this connection reference may be made to the seven series of analyses quoted by Pritchard (10), and the thirteen series quoted by Richmond (11). The average fat content of the present samples seems, however, distinctly higher than that of the figures quoted, which appear to range round the value of about 3·5% On the other hand the percentage of lactose in the present results seems to be lower than the average of many of the series of analyses quoted, which approaches 7·0%. The percentage of protein is also lower than many of the figures given.

In addition to the constituents mentioned above there is the ill-defined group of bodies known as the extractive substances. Camerer and Söldner (*loc. cit.*), who investigated these substances in human milk very thoroughly thirty years ago, found their average concentration ranged from 1% during the first month of lactation to 0·5% towards the end of lactation. Meigs and Marsh (8) give 1% as the average concentration. Denis, Talbot, and Minot (4) investigated the nitrogenous portion of these much more recently. They found it to consist of the same substances (the principal of which is urea) present in the same concentrations as the extractive nitrogenous materials of the blood. Their figures for total extractive nitrogen and urea agree substantially with those of Camerer and Söldner.

The mean, mode, and median of the calorific values of the samples are also given in Table 2. The figures are 76·9, 80·0, and 75·9 large calories per 100 grm.

of milk respectively. The values 9·2, 4·1, and 3·9 have been taken as the calorific values in the body of 1 grm. of fat, protein, and lactose.

When the variabilities of these figures for human milk are compared with corresponding figures for cows' milk (Wardlaw, 18), certain resemblances and differences are observed. As in the case of human milk, the freezing point is the least variable property; the lactose is the least variable constituent. The variability of the percentage of lactose in cows' milk is, however, about three times as great as that in human milk. The ash of cows' milk, on the other hand, the variability of which comes next to that of the lactose, is only about half as variable as the ash of human milk.

The fat and protein in cows' milk have about the same variability as in human milk. The amount of milk obtained at a milking is the most variable property of the samples in the case of cows' milk as well as of human milk, the variability being about the same in the two cases.

It seems rather remarkable that in several respects the milk of individual women is no more, and in some cases considerably less, variable in its properties than the milk of individual cows. It may safely be assumed that the conditions under which the cows lived were much more uniform than the conditions under which the women lived. In addition, the dairy cow is the result of a long period of selective breeding for the purpose of improving milk production. It would appear that the improved average milk production which can undoubtedly be brought about by this selection is not necessarily accompanied by a decreased variability of the product of the individual animals. Breeding has been successful in producing animals whose milk does not as a rule fall below certain specified values in certain respects, rather than a product whose properties converge to these values.

The relative variabilities of these results are also shown graphically (figure 1). In the figure the abscissae represent the percentage deviations of the different quantities from the median values. The scale for ordinates to the left of the median abscissa runs upwards; the scale for ordinates to the right of the median abscissa runs downwards. The curves obtained in this way are simply ogival curves, in which the ordinates have been measured in the usual way below the median, but in the reverse direction above the median. Instead of the usual S-shaped ogival curves, those obtained rise to a peak at the median. The steepness with which the peak is reached is a measure of the variability or dispersion of the results. The sharper the peak is, the less is the dispersion. The relative variabilities are more readily seen when the curves are plotted in this way than when they are in the ordinary ogival form.

These curves also avoid certain difficulties which are encountered when the variabilities are shown graphically by the frequency or probability curves plotted respectively by arranging the results in arbitrary groups, or by drawing the derived curves of the ogives.

Relation between Stage of Lactation and Properties of Milk.

In the following table the average values of the properties and composition of the samples obtained during different periods of lactation are shown. Four periods have been selected, so as to give groups containing comparable numbers of samples: from 3 to less than 8 weeks, from 8 to less than 12 weeks, from 12 to less than 16 weeks, and from 16 to 34 weeks. The first three groups cover monthly intervals (only one specimen occurring in the 3 weeks period). The last group covers a much longer interval.

TABLE 3.

Variation of Human Milk with Stage of Lactation.

Age of child (weeks) ..	3–7·5	8–11·5	12–15·5	16–34
Age of mother	27·5	25·5	27·6	27·5
Weight of child (kgm.) ..	4·36	5·15	5·75	7·14
Volume of sample (c.cm.) ..	62·7	71·0	63·3	66·7
Specific gravity (25°C./25°C.)	1·0302	1·0292	1·0295	1·0286
Depression of freezing point	0·585°C.	0·583°C.	0·581°C.	0·573°C.
Fat (per cent.)	4·66	5·29	4·54	5·17
Lactose anhydride (per cent.)	6·41	6·45	6·51	6·48
Ash (per cent.)	0·18	0·20	0·18	0·17
Protein (per cent.)	1·55	1·42	1·43	1·35
Number of samples, total ..	33	31	20	37

The variation of the average ages of the mothers of the above groups is only slight. This factor therefore introduces no complication into the variation due to the average ages of the children.

The average weights of the infants increase in a normal manner. The figures shown agree closely with the average figures for the two sexes given by Robertson (12) for South Australian infants of the same average ages.

The average volume of milk obtained from the breast shows no regular movement with the stage of lactation, but fluctuates slightly about the mean figure for the whole series of results.

The average specific gravities show a similar fluctuation about the mean value. But in this case the fluctuations are in the opposite direction to those of the volume.

The average values for the depression of freezing point fall as the stage of lactation becomes later. This fall is very slight between the third and sixteenth week of lactation, but more rapid between this period and the next. The range of variation, however, is only slightly over 1% of the mean value.

The average percentages of total solids and of fat fluctuate about the mean value in the same way as the average volumes of the samples, larger volumes being associated with higher percentages of total solids and of fat.

The average percentages of solids-not-fat fluctuate about the mean value in the opposite direction to the percentages of fat.

The average percentages of lactose show a rise as the period of lactation increases up to the sixteenth week, then a slight fall between this and the last period.

The figures of Denis and Talbot (4) show a much greater rise in the percentage of lactose during the course of lactation, and also higher figures than those of the present results. Many of their figures for the percentage of lactose exceed 8% after the tenth week of lactation.

From the end of the first month of lactation the percentage of ash varies in the opposite direction to the percentage of lactose.

The percentage of extractives shows only a slight tendency to fall during the period of lactation studied. The figures (calculated as described above) are as follow:

Week of lactation	4–8	8–12	12–16	16–34
Extractives (per cent.) ..	0·78	0·72	0·68	0·72

The values show little variation from the average for the whole period, which average is close to that observed by Camerer and Söldner (1) (*loc. cit.*).

The average percentages of protein fall slightly during the period of lactation shown in the above table. When these figures are considered in conjunction with those for the earlier stages of lactation (Wardlaw, 16, *loc. cit.*), the manner of variation of protein content with stage of lactation is shown more clearly. In the following table (Table 4) the average percentages of protein are shown from the first day of lactation to about the twenty-fifth week. The corresponding average weights of the infants are also shown where available.

TABLE 4.

Relation between Age of Child, Weight, and Protein in Milk.

Age.	Samples.	Body Weight. (kilograms).	Protein (per cent.).
0	—	3·56	—
1–2 days	3	—	3·30
3–4 ,, ...	39	—	2·33
5–7 ,, ...	36	—	2·06
7–11 ,, ...	24	—	1·86
3–7 weeks	33	4·36	1·61
8–11 ,, ...	31	5·15	1·42
12–15 ,, ...	20	5·75	1·43
16–34 ,, ...	37	7·14	1·35

The figures in the above table are plotted in the accompanying curves (Fig. 2). Periods of lactation are plotted as abscissae, while the percentages of protein and weights of the infants are plotted as ordinates.

The curve for the percentages of protein shows clearly the very rapid fall in the concentration of this constituent during the first week of lactation. During this period the values drop from over 3·3% to 1·86%. During the next five weeks the fall is less rapid, the value reached at the end of this period being 1·61%.

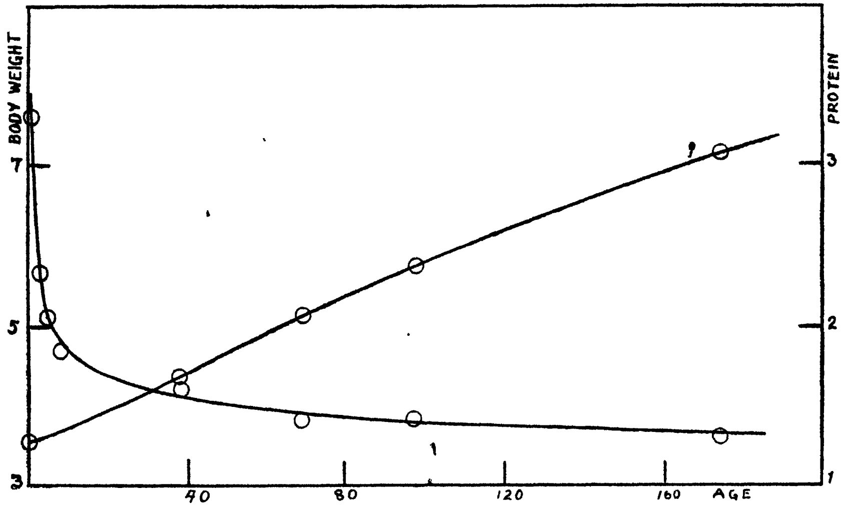

Figure 2.

Relation between percentage of protein in human milk and age of child (days), and between body weight (kg.) and age of child.

From this stage on the fall in the concentration is very slow. In the period between six weeks and thirty weeks the value decreases only from 1·61% to 1·35%. The percentage of protein may be said to reach a steady state after the first month of lactation.

The results differ from those obtained by one of us (Wardlaw, 18, *loc. cit.*) on the milk of individual cows. In the case of these animals the percentage of protein, after falling sharply during the early stages of lactation, rose again steadily, so that the concentration of protein was about 30% higher at the end of the thirty-sixth week of lactation than it was at the end of the eighth.

Variation with Age of Mother.

The following table shows the variations in the average properties of the samples of milk obtained from women between different ages. The ages have been placed in four groups, selected so as to give groups of samples comparable in number.

TABLE 5.

Variation of Human Milk with Age of Mother.

Age of mother (years) ..	18–22	23–26	27–31	32–43
Age of child (weeks) ..	10·9	13·8	12·1	12·0
Weight of child (kgm.) ..	5·32	5·65	5·76	5·63
Volume of sample (c.cm.) ..	60·7	65·6	67·0	71·2
Specific gravity (25°/25°C.)	1·0291	1·0291	1·0296	1·0297
Depression of freezing point	0·582°C.	0·587°C.	0·582°C.	0·582°C.
Total solids (per cent.) ..	13·95	13·98	13·51	13·45
Fat (per cent.)	5·13	5·30	4·65	4·63
Solids-not-fat (per cent.) ..	8·81	8·70	8·86	8·78
Lactose anhydride (per cent.)	6·45	6·44	6·47	6·50
Ash (per cent.)	0·19	0·18	0·17	0·17
Protein (per cent.)	1·51	1·37	1·48	1·41
Number of results, total ..	30	35	28	30

The average ages and weights of the children belonging to the women of the different age groups do not vary enough to introduce complications due to variations of stage of lactation.

The average volume of the sample increases distinctly as the average age of the subject increases. It must be borne in mind, however, that as the average age of the groups increases the proportion of primiparae which they contain decreases. It is usually more difficult to empty the breast by manual expression in the case of primparae than in multiparae. This circumstance might enable larger average samples to be obtained from multiparae of the older age groups, even if their milk secretion were not increased.

If the increase of the average volumes of the samples from the older age groups be due solely to more complete emptying of their breasts, a corresponding variation of the percentage of fat should be apparent. The fat content of the portions of milk last expressed from the breast are always higher than that of the preceding portions. The figures, however, show no parallel variation of the average volume and fat content, and give no indications that the difference is due to variations and the degree of emptying of the breast.

The average specific gravity is distinctly higher for the two older groups than for the two younger.

The average depression of freezing point is distinctly higher for the group between the ages of 23 and 26 than for the other groups.

The percentage of total solids varies in a direction opposite to that of the specific gravity; it is higher for the two younger groups and lower for the older groups.

The average percentage of fat varies in the same direction as the specific gravity, and is the cause of the variation of the latter. The milk of the two younger ages is distinctly richer in fat than that of the two older groups.

The average percentage of solids-not-fat does not vary in one direction, but fluctuates about the mean value for the whole series.

The average percentages of lactose and of protein also show no definite trend in one direction, but fluctuate about the mean values.

Relation between Number of Children Suckled and Composition of Milk.

In general it may be said that there is no obvious correlation between the ages of the women and the properties of their milk, other than that of the volume obtained from the breast. This increases with average age. There does not appear to be any very cogent reason for expecting any definite correlation between the properties of the milk and the age of the women except towards the extremes of the period during which lactation is possible. One would be more inclined to expect the mammary gland to reach and maintain a certain capability during this period.

When the properties of the samples are compared, not according to the ages of the mothers, but according to the numbers of times which they have suckled children, a relation appears between the number of children suckled and the volume of milk obtained from the breast, and also between the number of children and the weight of fat in the milk obtained. This is shown in the following table:

TABLE 6.

Average Volumes of Milk and Weights of Fat obtained from a breast of women who have suckled different numbers of children.

No. of Children Suckled	No. of Samples.	Age of Mother.	Volume of Sample.	Per cent. Fat.	Weight Fat.
1	65	24·7	63·7	5·16	3·29
2	24	26·8	66·7	4·84	3·26
3	17	30·4	71·3	4·68	3·34
4 and over	15	33·5	72·7	4·69	3·41

The average volume of the sample obtained from the breast increases with the number of children suckled by the subject. This increase is rapid at first, up to the third child suckled, and then becomes slower. The figures are plotted in the accompanying diagram (figure 3). This diagram reveals a tendency on the part of the volumes to approach a constant value in women who have suckled over four children.

These figures are in harmony with the view of MacFarlend (7), that a new set of acini develops in the mammary gland at each successive pregnancy. The constant volume which appears to be approached after a certain number of pregnancies is possibly that secreted when all the potential acini are actually secreting.

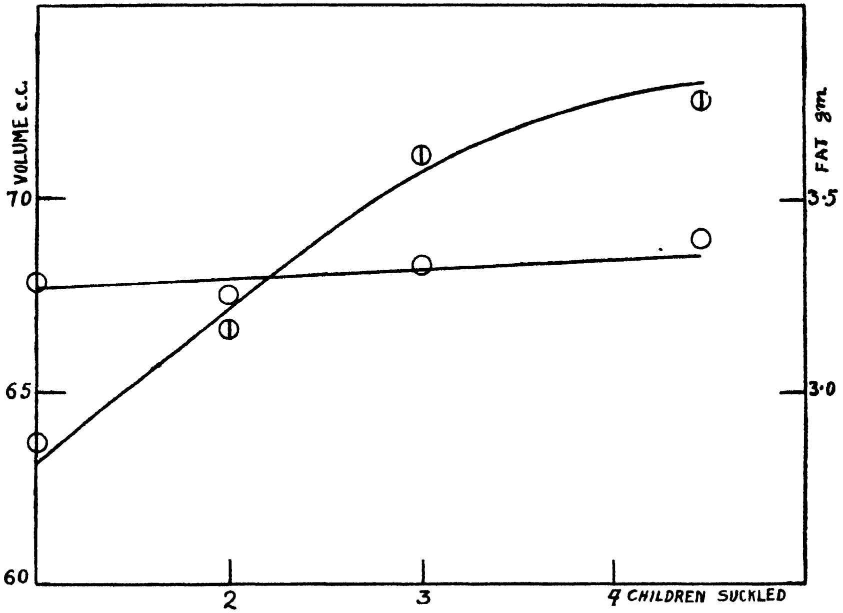

Figure 3.

Relation between number of children suckled, average volumes of milk obtained from breast Φ, and average weights of fat in the milk obtained O.

The figures for the weights of fat contained in each breastful of milk show quite a different variation with the number of children the women have suckled. There appears to be a very slight steady increase in the amount of fat in the samples of milk. This increase is so slight, however, that the weight of fat obtained from the breast can be said to show no material variation with the number of children suckled.

In other words, women tend to yield greater volumes of milk poorer in fat as the number of children suckled increases. The decrease in the percentage of fat is related to the volume of milk obtained in such a way that the weight of fat obtained is practically constant. The percentage of the substances other than fat shows no evident dependence on the number of children suckled.

Relation between Percentage of Ash and Percentage of Lactose.

Jackson and Rothera (6) have shown that there is an inverse relationship between the electrical conductivity of human and other milk and the percentage of lactose. A similar relationship should therefore exist between the concentration of ionized salts and the percentage of lactose, since the conductivity is determined by the former. The relationship will be modified by the fact that the conductivity is depressed by the protein content of the milk to the extent of nearly 3% for each 1% of protein present.

Figures for the concentration of ionized salts are not available for the present results, but when the average percentages of total salts or ash are compared with the corresponding percentages of lactose a similar inverse relationship is evident. This is shown in the accompanying table.

TABLE 7.

Relationship between Ash, Lactose, and Delta of Human Milk.

Lactose.	Lactose, Mean.	Ash, Mean.	Δ, Mean.	No. of Samples.
6·2% and under	5·84	0·205%	0·577°C.	11
6·2 6·45	6·38	0·186	0·580	50
6·45 6·7	6·55	0·179	0·582	45
Over 6·7	6·85	0·158	0·578	15

The similar relation which is to be seen when the average percentages of lactose corresponding to different percentages of ash are compared, is shown by the following figures:

Percentage of Ash	under 0·15	0·15–0·20	0·20–0·25	0·30 and over
Average % Lactose	6·68	6·51	6·40	6·28

The values of Δ have also been recorded in Table 7. They show no significant variation with the percentage of ash or of lactose, and demonstrate that variations in the concentration of lactose must be compensated for by inverse variations of the concentrations of soluble salts and other soluble matter.

When the percentages of ash and of lactose for the individual milks are compared the inverse relationship between the two is not shown so clearly as in the above table. This is no doubt due to the fact that the proportion of soluble to total ash varies considerably in the individual specimens (Michaelis and Rona, 9; Wardlaw, 19). In the average figures, on the other hand, the variations of the proportion of soluble ash in one direction are approximately compensated for by variations in the other direction.

Relation between Disturbances of Alimentary Canal and Composition of Milk.

The women whose milk has been examined in this investigation were in every case chosen as showing no material departure from normal, and giving no history of any recent serious illness. They were selected as suckling children who were also normal as far as could be determined, and for which no history of any serious illness was obtained.

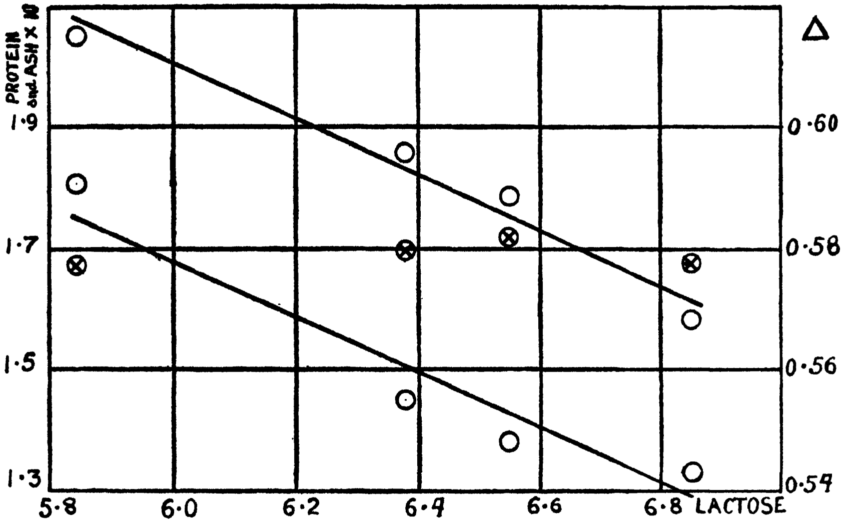

Figure 4.

Relation between percentage of lactose in human milk and percentages of ash (upper curve), percentage of protein (lower curve), and Δ (⊗)

In certain of the histories, however, reference was noticed to the child having had "diarrhoea and green motions." Six cases of this kind occur. The composition of the mothers' milk in these cases, and the ages of the mother and child, and the weight of the child are shown in the following table:

TABLE 8.

Relation between Composition of Mother's Milk and Alimentary Disturbance in Infant.

No.	Age of Mother. yr.	Age of Child. wk.	Wt. of Child. kg.	Sample Vol. of. cc.	Fat %	Protein %	Lactose %	Ash %
9	22	7	2.9	40	2.33	2.35	4.90	0.33
25	32	3	2.6	46	4.8	2.15	5.13	0.21
26	26	14	4.8	95	3.39	1.77	6.50	0.08
27	29	5	3.7	40	6.61	1.72	6.35	0.12
37	23	20	6.4	60	5.84	1.63	6.45	0.21
100	33	8	5.4	190	3.50	1.64	6.40	0.18
Mean	27.5	9.5	4.3	78.5	4.4	1.86	5.95	0.19

In each case the percentage of protein is above the average. The weights of the children are below the average, except numbers 37 and 100, the two lowest proteins of the series. The figures for lactose show a variation in the opposite direction to the figures for the protein.

Two of the above specimens (26 and 29) show exceptionally low percentages of ash, while two others (9 and 25) show exceptionally low percentages of lactose. The two latter specimens are those containing the two highest percentages of protein in the series. The first of them contains also an exceptionally high percentage of ash.

These figures are in general harmony with a widespread opinion that excess of protein in the mother's milk may be associated with alimentary disturbances in the infant. Carter and Richmond (2) many years ago published analyses showing this association. It should be observed, however, that in the four of the above cases in which the infants were under weight the mother's milk showed extreme variations in constituents other than the protein, and that many instances occur of percentages of protein as high as some of those in the above table in cases where there is no record of alimentary disturbance in the infant.

In conclusion, we wish to express our indebtedness to the staff of the Newtown Baby Clinic, without whose enthusiastic co-operation the bulk of this work would have been impossible. We are indebted also to the staff of the Tressilian Hospital, through whose good offices specimens of milk were obtained from several patients.

We also wish to express our thanks to Professor H. G. Chapman, in whose laboratory this work was done, for the interest he has shown in the investigation.

SUMMARY.

The modal values for the composition and certain properties of the milk of Australian women obtained between the first and ninth months of lactation are as follows: Depression of freezing point, 0·0583°C.; lactose, 6·45%; specific gravity, 1·0296; protein, 1·40%; ash, 0·19%; fat, 5·30%. These figures are in ascending order of the variability or dispersion of the quantities, which range from 0·0052 for the depression of freezing point to 0·195 for the percentage of fat.

The average percentages of protein and of ash, and the depression of freezing point, show a slight fall as lactation continues. The average percentage of lactose shows a slight rise.

The milk of the younger women is on the average distinctly richer in fat than that of the older women. The average volume of milk obtained from the breast varies in the opposite direction with age.

The average percentage of ash is slightly greater in the milk of the younger women, while the average percentage of lactose varies in the opposite direction.

The milk of the women between the ages of 22 and 27 has a distinctly greater average of depression of freezing point and smaller average percentage of protein than in the case of other age groups.

The average volume of milk obtained from the breast increases with the number of lactations up to the fourth. The average percentage of fat decreases, so that the average weight of fat obtained remains constant.

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OBSERVATIONS ON MITOCHONDRIA

by

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(Submitted for publication, 5th August, 1926.)

In this paper a further series of observations are reported concerning mitochondria of certain protozoa, which may help to elucidate the nature of these cytoplasmic inclusions.

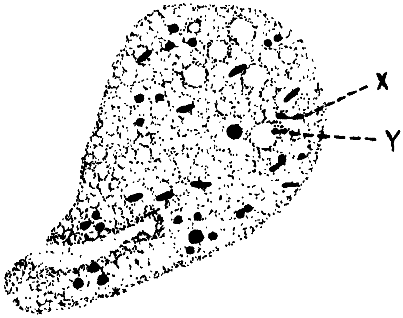
VITAL STAINING OF MITOCHONDRIA.

Experiments have been carried out on a new dye stuff, the sodium salt of diethyl safranine monocarboxylic acid, prepared from janus green by the hydrolysis of the nitrile, and kindly forwarded to me by Professor T. Brailsford Robertson. Dilute solutions (1 part in 5,000) have the colour of weak permanganate. With this substance, and at a dilution considerably greater than this, I have succeeded in staining the mitochondria in living paramoecia, and in an unidentified heterotrichan. The mitochondria are coloured a bright pink in about twenty minutes. In living amoebae they colour only very faintly, the organism encysting before the stain has had time to penetrate extensively. A heterotrichan in which the mitochondria have been thus rendered visible is shown in figure 1.

The stain does not appear to have any corresponding selective action on bacteria. This observation is of special importance, in view of the work of Wallin (9), who reports having succeeded in staining various living bacteria with janus green dilutions of 1 to 10,000. Tubercle bacilli stained very faintly, but certain unidentified bacilli and cocci were very intensely stained immediately the preparation was made.

In the parasite of Rocky Mountain spotted fever, described by Wellbach, Cowdry states, on the contrary, that symbiotic bacteria can be seen lying side by side with the mitochondria.

The stain employed in this investigation does not, however, lend any support to Wallin's contention. I find that in a culture of heterotrichan infusions, at the time when the mitochondria have been coloured a bright pink, bacteria may be observed in the surrounding fluid quite unstained, and still exhibiting active movement. It might be urged that these bacteria are covered with an impermeable membrane, which is absent in the mitochondria (assuming them to be symbiotic bacteria); but this seems to be disproved by the following observations. Mobile bacilli, after having been exposed for some hours to the stain, die; it seems justifiable to contend that the stain has penetrated them; nevertheless, they are not selectively coloured by it. I have tested this dye against the following bacteria, and after an exposure of six hours, and even long after it has killed them, it has not rendered them stainable: Bacillus coli communis, staphylococcus albus, and staphylococcus aureus.



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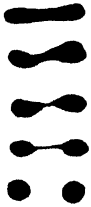

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Fig. 1. A Heterotrichan Infusion, showing mitochondria stained alive by "Janus red." Two of the mitochondria, indicated by X and Y, are seen undergoing binary fission. Some of the mitochondria are in the rod-shaped condition, others in the spherical.

Fig. 2. Various stages in the binary fission of the mitochondrium, indicated by X in the previous figure. The drawing was made from a mitochondrium which had absorbed the stain, and the process occupied four minutes.

There is a further difference between ordinary bacteria and mitochondria, namely, that bacteria exposed to absolute alcohol for several hours are still sharply stainable with iron haematoxylin, while mitochondria are rendered nonstainable, even after quite a short exposure. That mitochondria have a bacterial appearance cannot be questioned, but it is equally certain that they are not ordinary bacteria. That "mitochondria are symbiotic bacteria in the cytoplasm of all higher organisms, whose symbiotic existence had its inception at the dawn of phylogenetic evolution," as suggested by Wallin, cannot in the present state of knowledge be profitably discussed, and that they may be bacteria which have become incorporated into the evolving cell in a manner reminiscent of that developed in Mereschkowsky's and Minchin's speculation concerning cell evolution (6) is also at present beyond the bounds of scientific investigation. Whatever their origin, however, it seems very certain that they cannot at the present day be bacteria, for they now resemble bacteria only in external appearance.

Fission and Fusion of Mitochondria.

If mitochondria are examined in fixed and stained preparations, numerous conditions of these bodies are observed, which seem to indicate that they are undergoing binary fission. This was apparently first observed by Faure-Fremiet in certain protozoa. I have observed the same thing in Paramoecium (4) and Opalina (3), while M. R. and W. H. Lewis have figured apparently similar conditions in mitochondria observed in cells grown in tissue culture, and the occurrence of fission in mitochondria seems to be widely accepted at the present day. Similarly the occurrence of multiple fission in the long, filamentous mitochondria in the actively secreting pancreas cells seems clearly established. But the actual process of fission has not, so far as I am aware, ever been observed in living mitochondria.

In this paper I am able to report observation in which I have been able to follow the process of binary fission in living mitochondria. The observations have been made on mitochondria from a heterotrichan infusian stained *intra vitam* by the dye described above, and also on living unstained mitochondria from amoeba. The process was first seen in the two mitochondria indicated by X and Y in the heterotrichan shown in figure 1.

Different stages in the fission of this mitochondrium are shown at higher magnification in figure 2. The rod-shaped mitochondrium contracts slightly in the middle; the constriction then slowly becomes more marked, until finally two almost spherical bodies are formed, connected to one another by a thin filament; the filament then ruptures, and the two daughter forms round themselves off and become spherical. The whole process lasts four minutes.

In amoeba I have been able to observe fission. Since the observations have had to be made on entirely unstained specimens, the process has not been followed out with the same clearness as I have seen it in stained heterotrichan mitochondria. Nevertheless, of the actual occurrence of binary fission of rod-shaped mitochondria in other forms there can be no doubt. The best time to observe it is in the encysting forms of amoeba. An amoeba may be readily made to undergo encystment by placing it in an environment unfavourable to it, such as a weak janus green solution. Although the janus green does not extensively penetrate the organism, yet the latter rounds itself off, and in such forms numerous spherical bodies in pairs, and also many dumb-bell shaped forms, may be seen, evidently indicating an extensive occurrence of binary fission.

Fission of mitochondria accompanying encystment of the organism apparently occurs, therefore, in amoeba as it does in Opalina (3) and in Paramoecium (4).

Not only fission, but also fusion of mitochondria can be demonstrated in protozoa. An increase in size of mitochondria has been described by J. T. Scott (8); and Cowdry (2) believes that increase in size may result through a

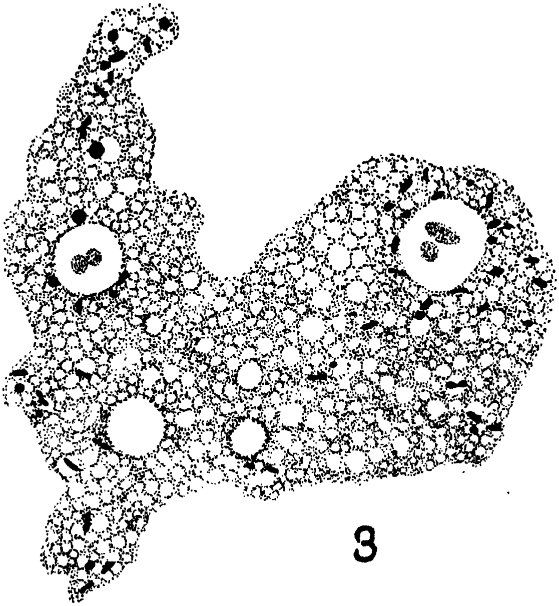

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Fig. 3. Living amoeba, showing mitochondria. Most of the mitochondria are in the rod-shaped condition; several are spherical. Two of the vacuoles contain food, but not mitochondria—they have evidently already dissolved.

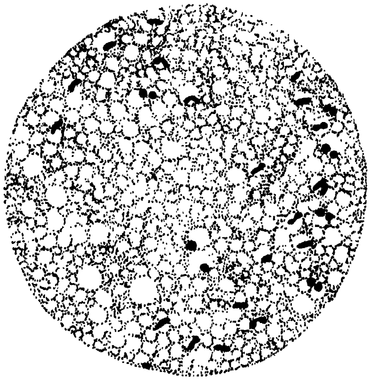

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Fig 4. An encysting amoeba, showing numerous mitochondria which are undergoing, or have just undergone, binary fission.

coalescence of two or more mitochondria. Similarly, W. J. M. Scott (7) has described the formation in the pancreas after phosphorus poisoning of large lipoid droplets produced apparently by fusion of mitochondria. I have also previously reported fusion of mitochondria in pancreatic cells, in cases when the pancreas has not been fixed immediately after killing the animal; and a similar fusion of mitochondria may be observed in opalina in cases when the organism has been allowed to live in Ringer's fluid for about twelve hours.

As, however, actual fusion of mitochondria has not been demonstrated in these cases, I may report an observation made on living amoeba, in which I have actually seen such fusion. In living amoebae many of the mitochondria are rod-shaped bodies; but frequently spherical forms also occur (such, for example, in figure 5 r). In the streaming cytoplasm of the living amoeba these highly refractive bodies are easily recognizable; they exhibit marked Brownian movement, and are constantly being swept about in the currents of protoplasm. On several occasions I have observed two such mitochondria always in the spherical condition, lying close together, and suddenly fusing with one another (see, for instance, figure 5 r). The fusion is probably a surface tension effect, similar to the fusion of oil globules, that may be observed in any suspension of oil in water, and it does not seem likely that any physiological function can be ascribed to it.

The Rôle of the Mitochondria in the Process of Intracellular Digestion. Observations on Living Amoebae.

Living amoebae are particularly favourable objects for the study of the behaviour of mitochondria, since these bodies are clearly seen as highly refractive bodies in the granular cytoplasm of the organism, and moreover, the movements undergone by the organism are so slow as to permit of leisurely observation.

In a previous paper (4) I have recorded an observation made on fixed and stained paramoecium, in which the food vacuoles were observed to contain rod-like bodies, similar to mitochondria in appearance, and which were not bacteria. These bodies gradually dissolve in the fluid contents of the vacuole, and since mitochondria are believed to be of an enzymatic nature, it was concluded that the process of extrusion of mitochondria into the food vacuoles constituted a visible passage of the digestive enzymes into the digestive vacuoles. We would here have a process of intracellular digestion, analogous to the process of extracellular digestion seen in ordinary glands, where the enzymes likewise appear to arise from the mitochondria.

In living amoebae I have been able to follow the whole processes of vacuole formation, and the relation of mitochondria to the process. When a foreign organism is engulfed by a living amoeba, the body circulates in the cytoplasm of the organism for a considerable time, lying in direct contact with the protoplasm,

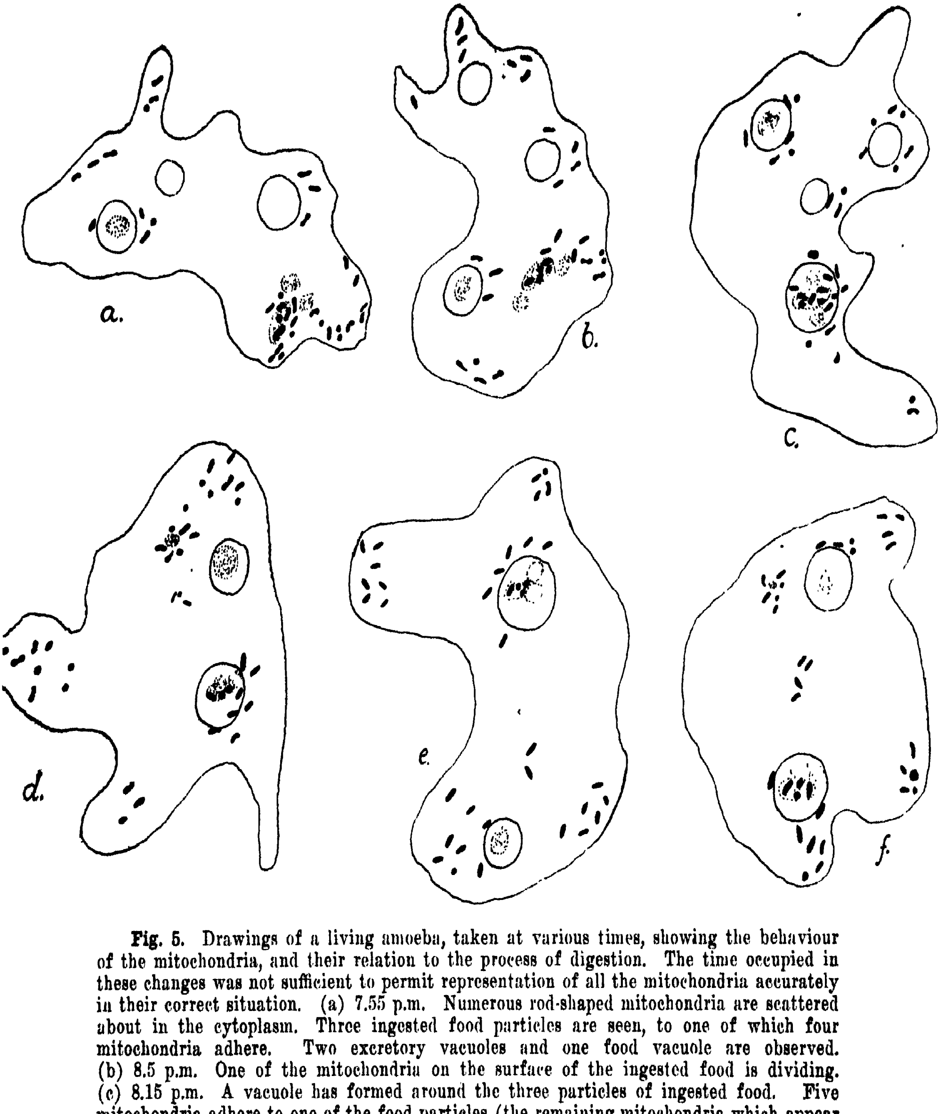

Fig. 5. Drawings of a living amoeba, taken at various times, showing the behaviour of the mitochondria, and their relation to the process of digestion. The time occupied in these changes was not sufficient to permit representation of all the mitochondria accurately in their correct situation. (a) 7.55 p.m. Numerous rod-shaped mitochondria are scattered about in the cytoplasm. Three ingested food particles are seen, to one of which four mitochondria adhere. Two excretory vacuoles and one food vacuole are observed. (b) 8.5 p.m. One of the mitochondria on the surface of the ingested food is dividing. (c) 8.15 p.m. A vacuole has formed around the three particles of ingested food. Five mitochondria adhere to one of the food particles (the remaining mitochondria which appear to lie within the vacuole are really above it within the cytoplasm). (d) 8.25 p.m. Another very small food particle has been engulfed, and is surrounded by several mitochondria. Its fate has not been followed out in the remaining figures. (e) 8.35 p.m. (f) 8.50 p.m.

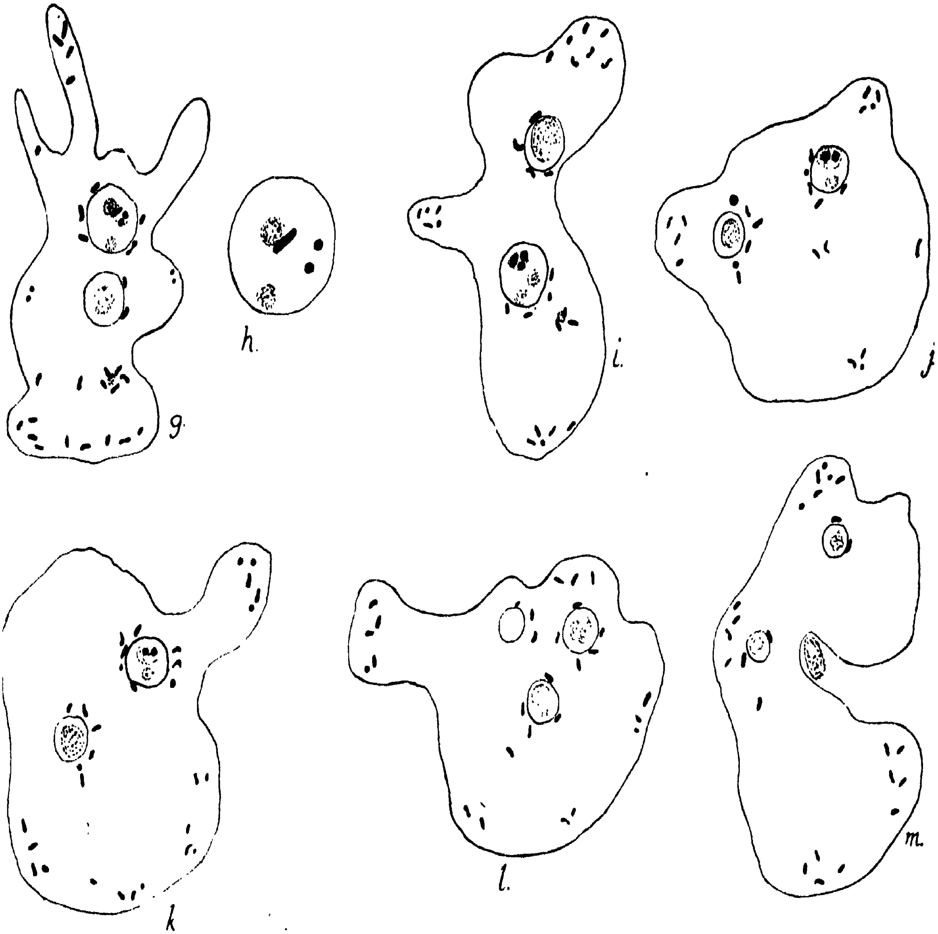

Fig. 6. (g) 9.15 p.m. Two of the mitochondria within the vacuoles have become spherical. (h) Food vacuole shown in the last figure at higher magnification. The spherical shape of the two mitochondria is very clearly seen. (i) 9.20 p.m. The mitochondria within the vacuole have rounded themselves off. (j) 9.35 p.m. One mitochondrium has been dissolved within the vacuole, and the vacuole has decreased in size. One of the particles of food has disintegrated and entirely disappeared. (k) 9.45 p.m. The mitochondria within the vacuole have decreased in size. (l) 9.55 p.m. The mitochondria have entirely dissolved and the vacuole has diminished greatly is size. (m) 10.16 p.m. One food particle has entirely disappeared, and the vacuole has decreased in size. The amoeba is at the same time engaged in engulfing a foreign organism (Euglena).

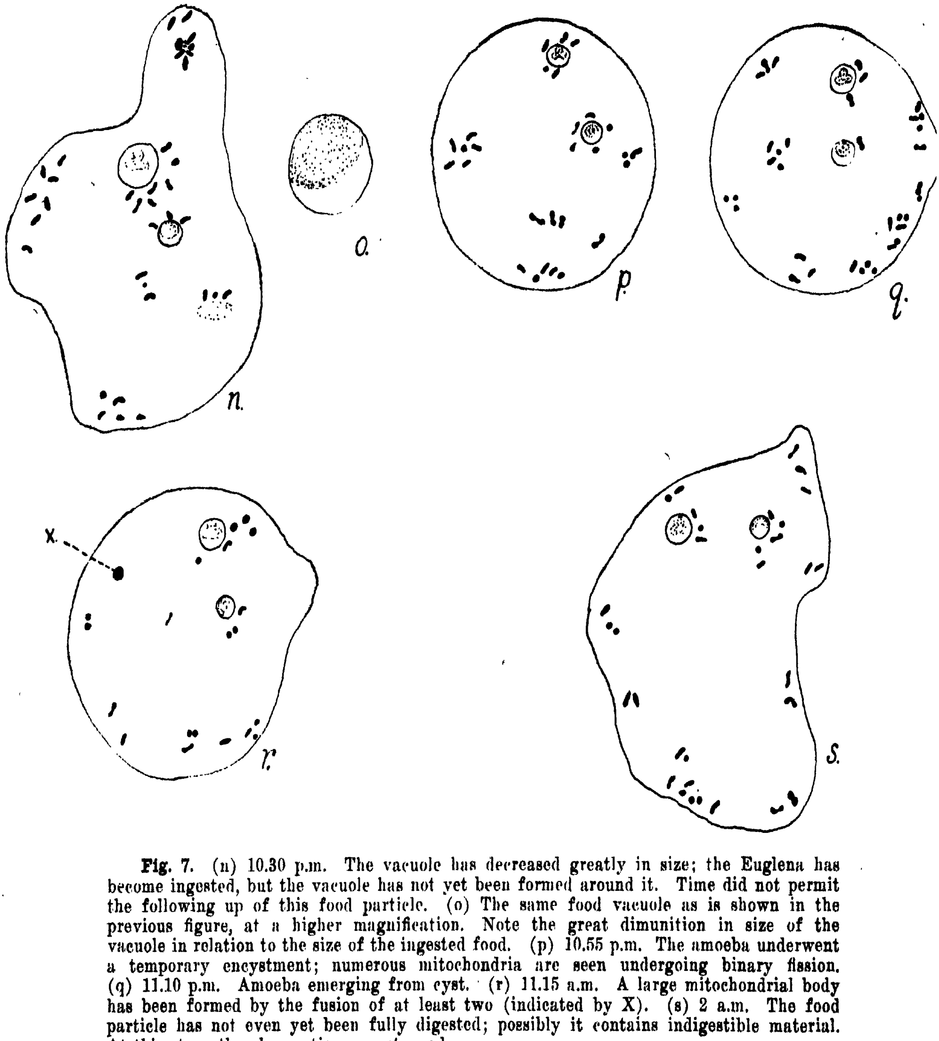

Fig. 7. (n) 10.30 p.m. The vacuole has decreased greatly in size; the Euglena has become ingested, but the vacuole has not yet been formed around it. Time did not permit the following up of this food particle. (o) The same food vacuole as is shown in the previous figure, at a higher magnification. Note the great dimunition in size of the vacuole in relation to the size of the ingested food. (p) 10.55 p.m. The amoeba underwent a temporary encystment; numerous mitochondria are seen undergoing binary fission. (q) 11.10 p.m. Amoeba emerging from cyst. (r) 11.15 a.m. A large mitochondrial body has been formed by the fusion of at least two (indicated by X). (s) 2 a.m. The food particle has not even yet been fully digested; possibly it contains indigestible material. At this stage the observation was stopped.

and not being separated from it by any visible vacuole. As this engulfed food moves about within the amoeba through the action of the streaming currents of protoplasm, it comes in close relation, frequently with the mitochondria which are scattered about in the organism. These mitochondria adhere to the engulfed prey. I have also seen cases where a narrow current of protoplasm moves past a stationary engulfed organism, and have seen a mitochondrium carried to the organism by this protoplasmic stream, and then observed the mitochondrium to adhere to the food, while the current swept past. By this means a small number of mitochondria come to adhere to the engulfed prey (see, for example, figure 5, a, b). It is, in fact, a common sight in living amoebae to observe an engulfed ciliate carried about in the protoplasmic currents, having attached to its surface a number of refractive bodies, the mitochondria. In this situation it may at times be observed that the mitochondria actually undergo fusion, and at other times fission.

After a number of mitochondria have adhered, probably merely through chance, to the surface of the food, a vacuole is gradually formed around the food particles. This condition is shown in figure 5 c, where at least five mitochondria are attached to the large food particle, and this food particle and two smaller ones are now contained in a single large vacuole. These vacuoles then slowly circulate about in the streaming protoplasm. At times the mitochondria may be observed to undergo binary fission within the vacuoles. After about half an hour the mitochondria suddenly disappear within the vacuole, having evidently become dissolved in the fluid contents of the vacuole (figure 6 l). The food at the same time seems gradually to disintegrate, and the vacuoles become very markedly reduced in size. The disintegration of the food is difficult to observe, and I have not been able to follow it to completion; but the reduction in size of the vacuole is very clearly seen, indicating probably an absorption of digested food (see figures 5, 6, and 7, l, m, n, o, p, q, r).

In a previous paper I described a preparation of paramoecium, in which a mitochondrium seemed to be actually passing through the wall of the vacuole. Whether this preparation really represented the entrance of a mitochondrium into the vacuole is, however, difficult to say. In amoeba I have never observed the extrusion of mitochondria into the vacuoles, but the mitochondria always adhere to the food particles first, and it is only subsequently that the vacuole is formed around the food particle. There seems to be good evidence in amoeba that the mitochondria cannot penetrate the membrane of the vacuole. In living amoebae, especially those with very large vacuoles, I have frequently seen mitochondria lying in the cytoplasm close against the walls of the vacuole. Here they always undergo typical Brownian movements; they may even indent the wall of the vacuole, but they never seem to be able to penetrate it. I have made this observation on various occasions, and have observed numerous such mitochondria lying closely against the vacuoles, but on no occasion have I ever seen them actually penetrate into their interior.

On the Number of Mitochondria which occur in Single Cells.

The statement has occasionally been made that the number of mitochondria are approximately constant within similar cells of similar species. For example, Wakelin Barratt (1) states that in the liver of the rabbit "The number of chondriosomes present in a single cell is approximately 70, the variations met with not appearing to be considerable." I have made counts of the number of mitochondria that occur in living amoebae, and there can be not doubt that in this organism the number varies greatly. For example, in seven amoebae the number present were: 17 (fairly large individuals), 28 (fairly large), 14 (fairly small), 15 (small individual), 30 (fairly large), 12 (small), 17 (fairly large). In this series there was certainly no indication of constancy of number of mitochondria, and one large individual even contained fewer mitochondria than another that was larger than itself.

Similarly, I find that in opalina, where the mitochondria are present in very large numbers, no indication of a constant number can be observed.

SUMMARY.

1. A dye-stuff (sodium salt of diethyl safranine monocarboxylic acid), formed from janus green by hydrolysis of the nitrile, is found to be selectively absorbed by mitochondria. Several bacteria have been tested with this substance, but they do not show a corresponding selective absorption.

2. Mitochondria of an heterotrichan infusorian, stained by this means, have been observed to undergo binary fission. The process occupies about four minutes. Fission of the unstained mitochondria is also described in amoeba.

3. In amoeba fusion of mitochondria has been observed.

4. Observations have been made in living amoeba on the relation of mitochondria to the process of intracellular digestion. The engulfed food circulates in the protoplasm, and mitochondria come into direct contact with it; they adhere to the food, and finally a vacuole is secreted around the food, with its adhering mitochondria. The mitochondria slowly dissolve, and the food undergoes disintegration. The vacuole slowly decreases in size, thus indicating an absorption of the digested food. It is concluded that the mitochondria, which are believed to be of an enzymatic nature, have brought about the digestion of the food.

5. Neither in amoeba nor in opalina does a constant number of mitochondria occur in the organism.

I wish to acknowledge my indebtedness to Dr. O. W. Tiegs, of Melbourne University, for his suggestions during the course of this investigation.

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FURTHER REMARKS ON THE STRUCTURE OF THE SPINAL CORD

by

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(Submitted for publication 26th July, 1926.)

The conception of successive neurones of a reflex arc, terminating the one upon the next, by branching, discontinuous synapses, which embrace the body of the nerve cell, or ramify among its dendrites, has become so generally accepted, mainly through the histological work of Ramon y Cajal, that the new interpretation which I have placed upon the structure of the neurone junctions (2, 3) may deserve further discussion.

The conclusion previously reached, was that the union of the neurones took place through the cell dendrites; this does not, of course, imply a dendrite anastomosis of the kind described many years ago by Gerlach, but on the contrary considers that the fine collaterals that pass from the white matter into the grey matter, enter the dendrites of the nerve cells either singly or in bundles, so that the integration will occur within the body of the nerve cell, and not on a synaptic membrane which is closely applied to the nerve cell.

The only convincing illustration of a synapse within the spinal cord is the well-known figure of Cajal (see Cajal's book, vol. I, figure 110), of three nerve cells of Clark's column, having each a large, branching, terminal arborization from a collateral spread over its surface. The preparation was made by the defective methylene blue process, a method which, as I have previously pointed out, yields some very confusing artefacts. The only other evidence for the existence of synapses within the spinal cord consists of certain illustrations of small conical "knobs," closely applied to the body of the nerve cells. But little weight can be attached to this observation, since the "knobs" have never been shown to be connected with nerve fibres.

The whole question of the structure of the neurone junctions of the cord may best be understood by reference to the accompanying figure of a transverse section through an entire half of the spinal cord. The figure has been drawn from a section of the cord 25μ in thickness, and stained by the reduced silver method of Cajal, using alcohol as the fixative, and hydroquinone as the reducer.

The preparation was an extremely successful one, the whole of the nerve cells, with their axons and dendrites being completely stained. The completeness with which the staining has taken place is best appreciated by observing the white matter, in which an enormous number of closely packed longitudinally running nerve fibres have been blackened by the stain. These fibres, as the figure shows, are of varying thickness, and fibres of the same thickness usually tend to run together. Furthermore, if we follow any of the axons or "dendrites" from the various nerve cells in the grey matter, we can often trace them over very great distances, and when such axons or "dendrites" end, their ending is really a false one, for they have merely been cut short as they emerge from the plane of the section, and no true axon or dendrite termination can be observed. This fact is very readily made out by focussing the microscope up or down at the point where the fibres end, when such ending will invariably be found to be accompanied by the fibres passing out of the section. Since no natural termination of axons or "dendrites" can be demonstrated, we are justified in concluding that they have become very completely stained. Finally, it is possible to conclude that practically every nerve cell has been stained. Incompletely stained preparations always show numerous unstained nerve cells, obviously recognizable through the appearance of their nuclei, such nuclei being very clearly distinguishable even when they are quite unstained. In the preparations from which the accompanying illustration was drawn, only an occasional unstained nerve cell occurred. Finally, although the nuclei of the neuroglia cells have stained (they are not included in the figure) the neuroglia fibres have not been rendered visible, so that no opportunity has arisen for confusing fibres of neuroglia cells with nerve fibres. From these considerations, then, we are justified in concluding that the entire system of nerve fibres, or nearly the entire system, within the section, has been rendered clearly visible by the stain.

It is customary to represent "synapses" in two different ways: either as branching endings of nerves that ramify among the dendrites of a nerve cell, or as branching endings that spread over the "body" of the nerve cell, and embrace it closely, but do not come into direct contact with it.

The former mode of representation of a synapse is found in most diagrams illustrating the structure of the reflex arc of the spinal cord, and Cajal himself gives schematic figures of synapses conceived in this manner (see Cajal's book, vol. II, figure 549). The impossibility of this view is best illustrated by reference to the accompanying figure of the section through the spinal cord. In many of the nerve cells, especially those of the anterior horn, the "dendrites" can be followed for very long distances away from the "body" of the nerve cell, and some of the nerve cells spread over half the area of the section. The synapse, conceived as a branching nerve termination, ramifying among the "dendrites" of the nerve cell, would have to be of similar dimensions. As no histologist has

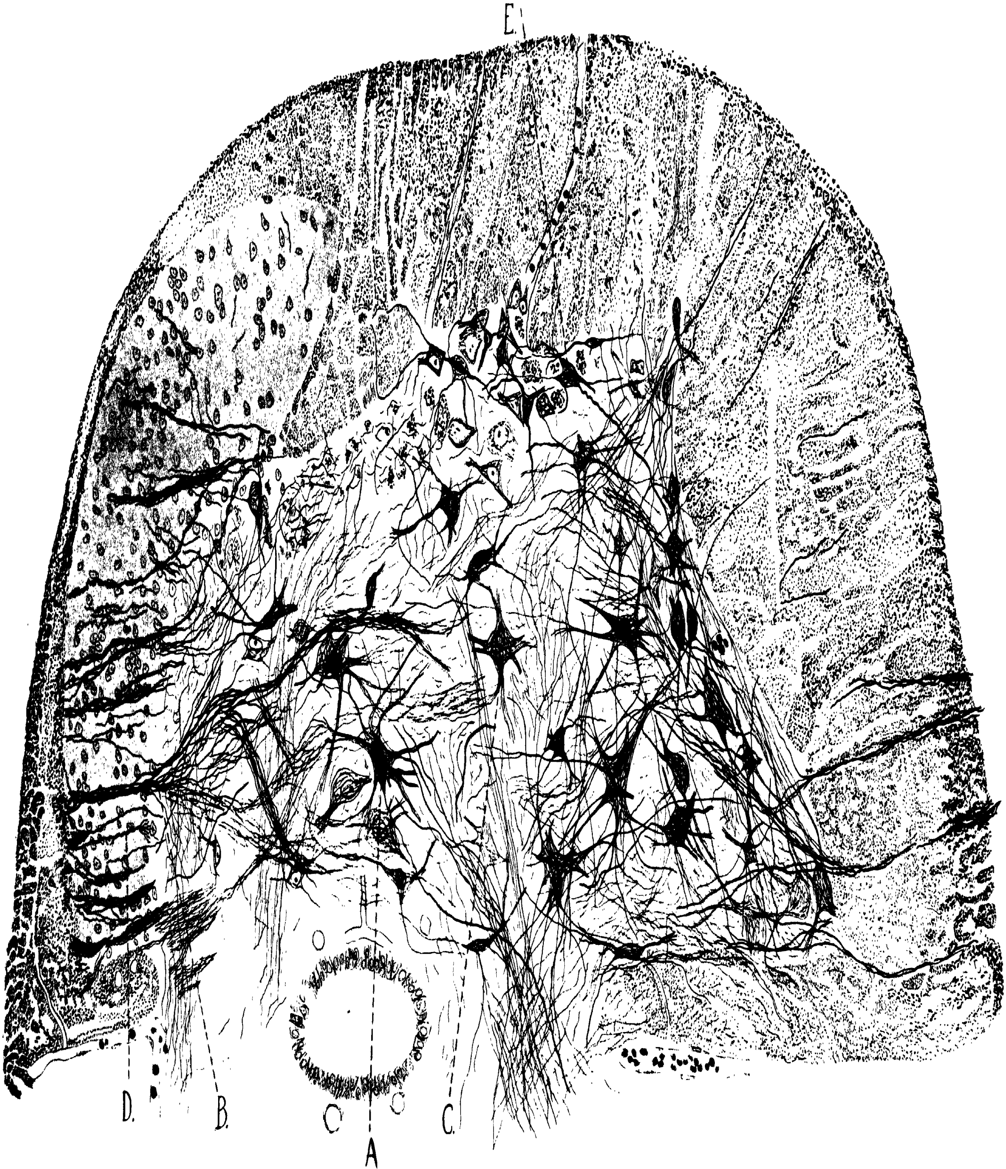

Figure 1. Transverse section through half the spinal cord of a new-born rabbit, in which the whole of the nerves seem to have been stained completely. In the outer white matter enormous numbers of transversely cut, longitudinally running fibres of varying thickness are seen. From the white matter collaterals may be seen passing into the grey matter, especially through the substance of Rolando on the dorsal side. Small scattered bundles of longitudinal fibres are also seen in the outer regions of the grey matter. A large bundle of collaterals enters the grey matter at B, and some of its fibres communicate (without the formation of synapses) with various nerve cells. Most of the collaterals have been cut short during the preparation of the section [illegible] the grey matter cannot be shown to have any relation to nerve cells, since they pass rapidly in and out of the section. The dendrites of the nerve cells are very short, being generally shorter than the nerve cell itself; into the dendrites the fine fibrils from the grey matter are seen to pass, and in some cases they may be traced away from the nerve cells for very long distances. On none of the nerve cells can branching synapses be seen. In some of the nerve cells the axon is clearly recognizable, and can be followed for very long distances (e.g. the nerve cells indicated by A and E). Occasional blood vessels are also recognizable. Ramon y Cajal's Reduced Silver Method.

ever claimed to observe such a structure, it is safe to say that this mode of representing synapses is entirely unjustified.

As evidence for the second mode of representing a synapse, we have, as far as I am aware, a single figure, that drawn by Cajal from a methylene blue preparation (see Cajal's book, vol. I, figure 110). In this well-known figure several collaterals are shown, terminating by branching arborizations on nerve cells from Clark's column. But a possible alternative explanation for this figure is, as I have pointed out before (3), that methylene blue often yields serious coagulation artefacts, and such artefacts, when represented on paper, may be indistinguishable from branching synapses. On the other hand, the section through the spinal cord here given, leads to an entirely different conclusion. The drawing is from a reduced silver preparation, in which, as discussed above, practically the whole of the neurones seem to have stained: a denser mass of fibres than that shown in the figure could scarcely be expected. Nevertheless, on not one of the many nerve cells can a single synapse be observed. The experimental work of Sherrington has shown that when a neurone ends upon another neurone of a reflex arc, numerous other neurones must also converge upon this second neurone, so that this second neurone will be the servant of not one, but of many neurones afferent to it (Sherrington's Principle of Convergence). If Cajal's interpretation of a synapse were correct, we should be justified in expecting the preparation, the *whole of whose nerve elements have stained*, to show a dense cluster of branching synapses embracing the bodies of all the nerve cells. But not the slightest indication of such a cluster can be detected. On the contrary, the fine fibres which pervade the grey matter enter the dendrites of the nerve cells singly or in small bundles, and having entered the cells, become continuous with, and are indeed indistinguishable from, the neurofibrils of the nerve cell. This has been clearly illustrated in my two previous papers, and need not be further discussed here.

In attempting to verify these observations on preparations of the spinal cord; instances will often be observed of nerve fibres that end fairly close to the nerve cells. But careful examination will invariably show that they are only fibres which have been cut short as they left the plane of section of the preparation.

A more serious objection might be raised against the conclusions here reached, namely, that although it is often possible to trace the fine fibrils outwards from the nerve cell, sometimes for very long distances, yet it is seldom possible to trace them into the white matter; are we therefore justified in regarding them as neurones that have grown into the nerve cells, or must we consider them, as Cajal did, merely as enormously long dendrites that have grown out from the nerve cells, and extend over long distances in the cord? It is in the failure to recognize this distinction that the whole misinterpretation of neurone junction structure in the cord has arisen. Firstly it is to be noticed that when we trace the fibrils outwards from the nerve cells they are never observed to have any actual termination, but their ending is always due to their leaving the plane of the section; we should have to conclude that the "dendrites" were of indefinite length. Secondly, it often happens that we can trace one of the fibrils into the white matter, but it is only rarely that we can do this for more than one fibril; but when we observe the irregular path that the fibrils take as they travel through the grey matter it is obvious that it is only an occasional fibril that we might reasonably hope to trace for any considerable distance. Still fewer nerve cells would be found in which we could trace more than one fibril right into the white matter. In such sections of the spinal cord, indeed, we can seldom trace even the axons for any great distance; nor is it usually possible to trace any individual collateral far from the white matter into the grey, and, as we might expect, we also find in the grey matter numerous fibres which cannot be shown to have any relation whatever to nerve cells; but in all such cases, examination under high magnification shows that the fibres have been cut short during the preparation of the sections, and that their endings are only artificial. Thirdly, if the fine fibrils were merely cell dendrites that have grown out from the nerve cells, we might expect them to have the appearance of protoplasmic outgrowths from the nerve cells, the cell membrane that covers the nerve cell being continued over them just as this membrane covers the axon that grows out of the cell. But if well-stained nerve cells are examined it will be observed that such a cell membrane is absent; the preparations, indeed, seem to show that these fibrils have either grown into the nerve cell from without, or that they have been formed by outgrowth from the neurofibrils of the nerve cell, but not that they are enormously elongated outgrowths from the walls of the body of the nerve cell. This fact is made very clear by employing the reduced silver process, but it is impossible to reveal it by the cytologically crude Golgi method, and it is on this latter method that Cajal has in the main relied for his elucidation of the structure of neurone junctions.

From these considerations we must conclude that, while it is not usually possible to trace the fibrils that appear to grow into the nerve cells, outwards from the white matter, from which they enter the grey matter, yet it is equally impossible usually to trace individual undoubted collaterals very far into the grey matter, since their path so seldom approaches that of a straight line. Nevertheless, these collaterals must have some relation to the nerve cells of the grey matter; and when we observe fine fibrils (though they cannot usually be traced individually into the white matter) approaching the nerve cells as components of bundles of fibres (many of which are undoubtedly neurone collaterals) and when we further observe that these fibrils that now enter the dendrites of the nerve cells are identical in appearance with undoubted neurone collaterals, or branches of these neurone collaterals; and, lastly, when we find that branching synapses applied to the body of the nerve cells do not occur, we seem justified in concluding that the fine fibrils that enter the nerve cells by way of its dendrites are identical with neurone collaterals. For example; in the accompanying figure the nerve cell indicated by "A" receives numerous fine fibrils by two of its dendrites from bundles of fine neurone collaterals that enter the cord through the substance of Rolando. We cannot trace these fibrils that have entered the dendrites outwards right into the white matter, for they leave the plane of the section, nevertheless, we seem justified in concluding that they are branches of the neurone collaterals that penetrate the substance of Rolando at B, for they are members of a bundle of fibres, many of which undoubtedly originate from the collaterals, and in appearance, moreover, are quite indistinguishable from these. The nerve cell at C receives fibrils from this same bundle, but it would be even more unreasonable to expect to trace these fine fibrils right into the white matter.

On Nerve Dendrites.

In reduced silver preparations the dendrites of the nerve cells are seen as thick outgrowths from the body of the nerve cells. These dendrites are of varying thickness and length, being sometimes short, at other times as long as the body of the nerve cell itself, and may even at times undergo a limited amount of branching. Into these dendrites the fine nerve fibrils pass, and usually no difficulty is experienced in distinguishing between dendrites and the fibrils that have grown into them. But in Golgi preparations the whole nerve cell, with its dendrites and fibrils, appears uniformly black, and we get the false appearance of nerve cells devoid of internal differentiation, and provided with enormous dendrites, that may sometimes extend nearly over the whole section of the cord. Even in reduced silver preparations, if these are too strongly stained, a somewhat similar effect may be obtained, and, indeed, in some of the anterior horn cells shown in the figure, apparently undifferentiated "dendrites" may be observed.

If we observe the general disposition of the true dendrites of the nerve cell we notice that they are not disposed irregularly and at haphazard within the grey matter, but on the contrary are in the direction of the small bundles of collaterals that traverse the grey matter, giving the appearance of processes that have grown out from the nerve cells in order to "collect" fibrils from various regions of the grey matter into the body of the nerve cell. In the nerve cell indicated by A this tendency is obvious; but usually the paths of fibrils are so irregular within the grey matter that the tendency to arrange themselves along the fibril paths becomes much obscured. This arrangement is especially difficult to represent in the drawing, since so much of it necessarily occurs in planes angular to the plane of the drawing. It is usually best observed at the edge of the grey matter, where well-defined fibril paths exist. Such a case is shown in figure 3, taken from a posterior horn cell, closely adjacent to the substance of Rolando. Three bundles of collaterals are seen entering the grey matter, and the dendrites are disposed in the direction of these fibrils; some of the fibrils enter the nerve cell, but most of them pass over the nerve cell, and end short, being cut across as they left the plane of the section.

The difficulty of observing the relation between the collaterals and the nerve cells to which they are related is increased by the fact that the collaterals, shortly before entering the grey matter, may undergo considerable branching. This, at any rate, is the case in the dorsal fibres in the cord. The fibres that enter the grey matter from the white matter usually do so in massive bundles, so that it is not usually possible to observe to what extent branching of these fibres has occurred. Nevertheless, occasional fibres, if not obscured by others, seem to show branching very clearly; see, for example at D in the figure. This branching collateral is shown at higher magnification in figure 2. (Incidentally it may be noticed that this branching fibre has the appearance of a synapse. The resemblance is a superficial one, for the termination of the fibres is a false one, due to the fact that its fine branches are cut through in making the section, while in any case it "terminates" in the substance of Rolando, in which nerve cells do not occur.) The extensive branching undergone by the dorsal collaterals as they pass through the substance of Rolando seems to be indicated by the thick mass of very fine and irregular fibres that lie at the border of this substance and the adjacent portion of grey matter. A similar branching of dorsal collaterals is shown in many of the figures of spinal cord sections given by Cajal from preparations made by the method of Golgi.

SUMMARY..

A drawing is given of a complete transverse section through half the spinal cord, prepared by the reduced silver method of Cajal. In this preparation, in which, it is believed, the *whole* of the neurones are completely stained, no trace of branching synapses applied to nerve cells, or ramifying among their dendrites, can be detected. On the contrary, the collaterals, probably after undergoing branching, penetrate the dendrites of the nerve cell, and become continuous with its neurofibrils in the manner described in two previous papers (2, 3).

The dendrites of the nerve cells are so disposed as to enable them to "collect" into the nerve cell individual fibres from the bundles of collaterals that traverse the grey matter.

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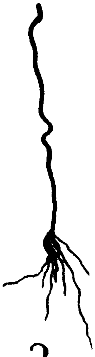

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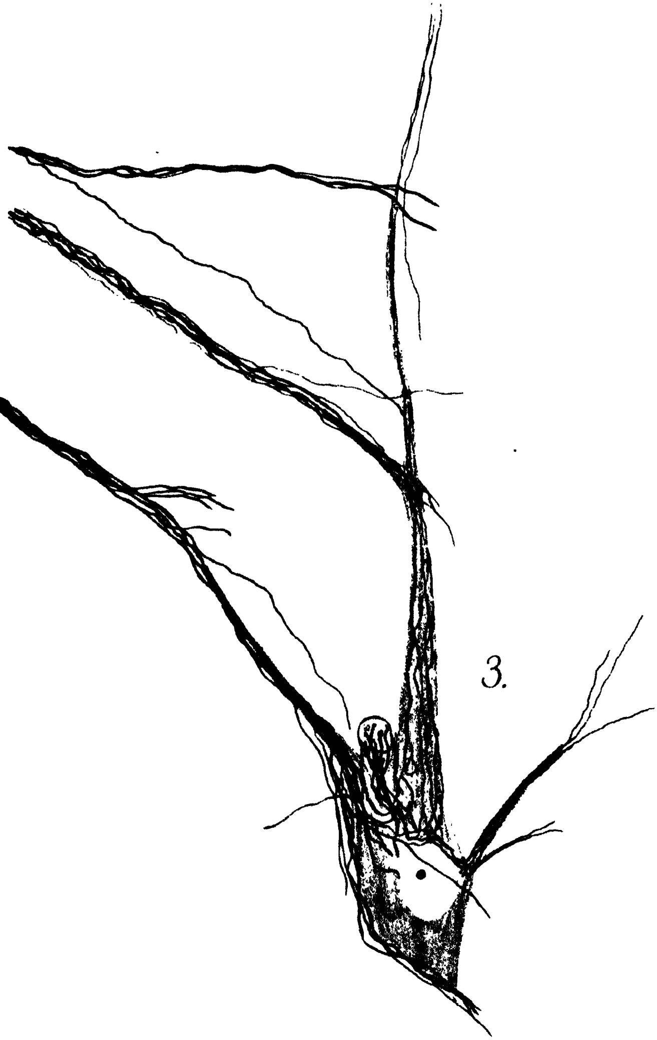

3.

Figure 2. A posterior collateral, undergoing branching. This is the collateral shown at D in the previous figure.

Figure 3. A nerve cell from the posterior horn, with dendrites disposed in the direction of three bundles of collaterals, some fibres of which penetrate the dendrites, though most of them pass over them.

Rabbit. Cajal reduced silver method.

VARIATIONS IN THE UREASE CONTENT OF DIFFERENT VARIETIES OF SOYA BEAN *(Glycine hispida)*

by

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(Submitted for publication 30th June, 1926.)

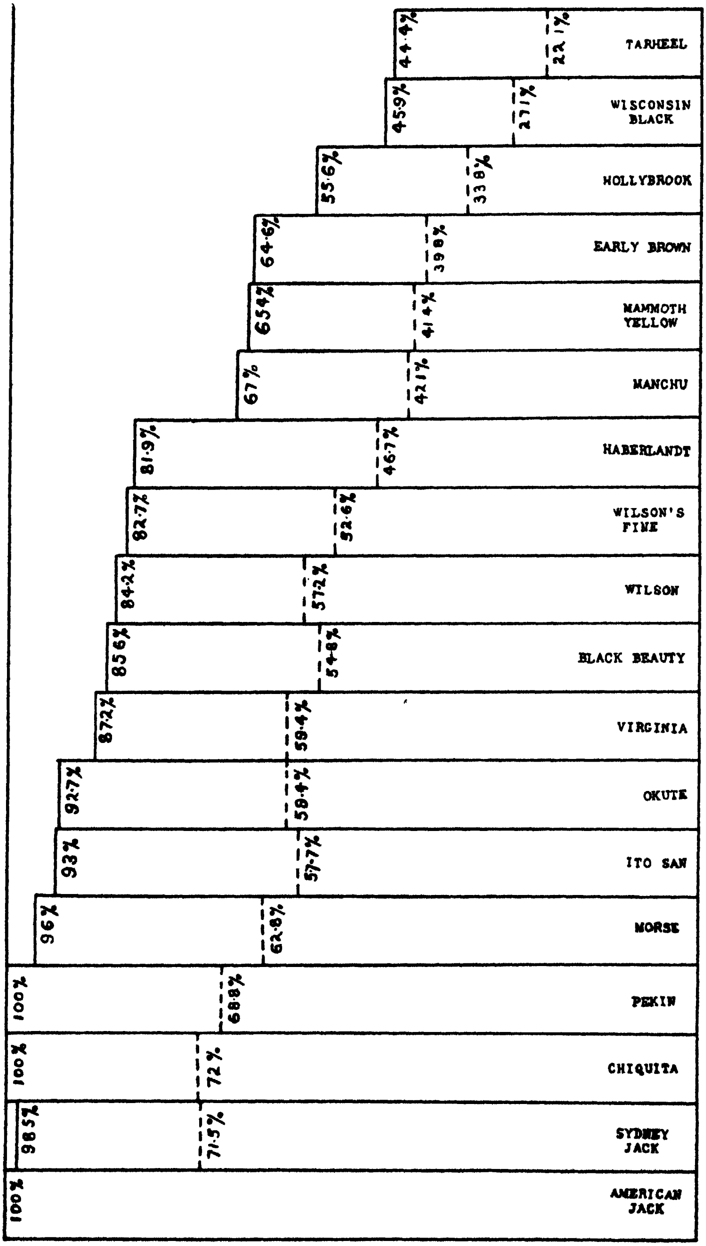
Since Takeuchi discovered the presence of urease in the soya bean this bean has been employed extensively for the detection and estimation of urea, particularly in urine and in blood. The urease converts the urea into ammonium carbonate quantitatively, and the ammonia can then be estimated by suitable means.

Annett (1914), working in Bengal, India, tested several varieties of soya beans, in order to see if there were any large variations in their urease activity. He distinguished these beans by the colourings of the seed coats, namely, soya bean yellow, soya bean spotted, soya bean chocolate, soya bean common, soya bean black, and Rymbsa Ktang, and he found that five of these six varieties were practically identical in urease activity; soya bean spotted was found to be distinctly more active.

Mateer and Marshall (1916) determined the relative amounts of urease in a number of different beans, and showed that the Jack bean *(Canavalia ensiformis)* contained about fifteen times as much urease as their soya bean. They also found that the sword bean *(Canavalia gladiata)* was much more active than the soya bean, but not as active as the Jack bean.

Other seeds and plant parts have since been examined, but there does not seem to have been any further investigation as to the relative urease activity of different horticultural varieties of the same bean.

For the determination of urea in this laboratory a Jack bean meal from America has been used, but since one lot of this deteriorated, and stocks were low, it was necessary to seek some other source of urease. The locally grown soya bean was obtained, and it was found that many varieties of soya bean are grown, differing in size and shape of the seeds and in the colour of the seed coat. Owing to the kindness of the manager of the Glen Innes Experimental Farm, Mr. C. S. Sparks, and of Mr. Carne, samples of the seeds of sixteen varieties were obtained and examined. These may be classified as black, brown, yellow, and



Graph representing the % urea decomposed by the bean extracts in a 1% urea solution at 51°C.

—— represents % urea hydrolyzed in 60 minutes.
- - - - represents % urea hydrolyzed in 30 minutes.

yellowish green, from the colour of their seed coats. Table 1 gives the names of the varieties and their colours.

TABLE 1.

Black.	*Brown.*	*Yellow.*
Pekin	Virginia	Chiquita
Wilson	Early Brown	Mammoth Yellow
Wilson's Fine		Manchu
Black Beauty	*Yellowish Green.*	Ito San
Wisconsin Black	Morse	Hollybrook
Tarheel	Okuter	Haberlandt

These beans were all grown on the same field, on the same kind of soil, at the Glen Innes Experimental Farm, and were all gathered shortly before they were examined, so the differences observed cannot be due to differences in the conditions of growth nor in the freshness of the seed.

To determine the urease activity of the beans the effect of extracts on a 1% solution of urea was observed.

To make the extracts 10 grms. of the finely-ground beans were added to 100 cc. of 30% alcohol, the mixture shaken from time to time, allowed to extract over night, and then filtered. Faintly opalescent extracts were obtained. In making the extracts 30% alcohol was used instead of water, since the solutions filtered so much more readily. Further, Marshall (1914) has shown that 30% alcohol does not appreciably retard the reaction between the urease and urea, nor is there much apparent destruction of the enzyme by alcohol of this strength.

To carry out the estimations 10 cc. of the filtered extract were added to 100 cc. of a 1% solution of urea, previously brought to the temperature at which the experiment was being conducted. The mixture was kept in small stoppered bottles, in order to reduce the likelihood of loss of ammonia. At intervals 5 cc. were removed as quickly as possible, and run into about 100 cc. of cold water. This was then immediately titrated with N/10 sulphuric acid, with methyl orange as indicator.

Experiments were carried out at 51°C. and 15°C., the bottles being immersed in a water-bath with a constant temperature regulator. Duplicate estimations gave good agreement.

TABLE 2.

	15 mins.	30 mins.	45 mins.	60 mins.
American Jack ..	94·0	100	100	100
Sydney Jack	43·2	71·5	88·0	98·5
Chiquita	42·7	72·0	93·6	100
Pekin	36·8	68·8	92·8	100
Morse	37·2	62·8	84·6	96

	15 mins.	30 mins.	45 mins.	60 mins.
Ito San	32·4	57·7	76·5	93
Okute	34·6	59·4	75·2	92·7
Virginia	35·0	59·4	75·2	87·2
Black Beauty	31·6	54·8	74·1	85·6
Wilson	37·2	57·2	72·2	84·2
Wilson's Fine	31·6	52·6	70·3	82·7
Haberlandt	29·3	46·7	69·5	81·9
Manchu	25·6	42·1	53·4	67·0
Mammoth Yellow	24·8	41·4	54·1	65·4
Early Brown	24·1	39·8	52·6	64·6
Hollybrook	18·8	33·8	44·4	55·6
Wisconsin Black	16·5	27·1	37·6	45·9
Tarheel	15·0	22·1	36·8	44·4

Percentage of urea decomposed by bean extracts in a 1% urea solution at times stated at 51°C.

Tables 2 and 3 give the percentages of urea decomposed at the times stated, when the temperatures were 51°C. and 15°C. respectively.

It will be seen from Table 2 that at 51°C. there is considerable variation in the urease activity of the extracts of different varieties of soya bean. In a period of thirty minutes the amount of urea decomposed varied from 22·1% to 72%. In an hour extracts of two of the varieties only had hydrolyzed the whole of the urea present in the solution, while the extracts of the other varieties hydrolyzed from 44·6% to 96% of the urea.

For comparison some of the Jack bean meal received from America, which had been in stock for some twelve months, and some Jack beans recently grown in Sydney were examined in the same way.

The American Jack bean was found to be very much more active than any of the soya beans, while the locally grown Jack bean was no better than the best of the soya beans.

To what the differences in the urease content of the different varieties of soya bean are due is not clear, but the fact that such great differences do occur is of interest, and makes it clear that before using a sample of soya bean or of Jack bean for the estimation of urea its activity should be determined.

TABLE 3.

	2 hours.	3 hours.	4 hours.	5 hours.	24 hours.
American Jack	59·8	69·0	83·0	91·0	100
Sydney Jack	41·0	54·0	62·0	71·0	100
Chiquita	36·6	45·5	54·5	63·4	100

	2 hours.	3 hours.	4 hours.	5 hours.	24 hours.
Morse	33·2	43·3	52·0	60·4	100
Pekin	30·6	40·3	47·8	56·0	100
Wilson	25·7	34·3	42·6	50·0	100
Wilson's Fine ..	25·4	34·3	41·0	50·0	100
Wisconsin Black ..	23·5	31·3	35·5	45·7	77
Tarheel	21·0	23·9	27·6	32·5	70·5

Percentage of urea decomposed by bean extracts in a 1% urea solution at times stated at 15°C.

Table 3 shows the activity of a number of the bean extracts at room temperature (15°), and these experiments, like those at 51°C., show that there are considerable differences in the urease content of the various beans.

The amount of urease hydrolyzed varied for the different soya beans from 32% to 71·1% for a period of five hours.

At room temperature (15°C.) the activity of the urease is markedly lower than at 51°C., and at the end of five hours, even the much more active Jack bean had not decomposed the whole of the urea present in the solution, whereas at 51°C. it had converted the whole of the urea into ammonium carbonate within half an hour.

SUMMARY.

The urease activity of eighteen varieties of soya bean was determined, and considerable variation was obtained.

At 51°C. 1 grm. of urea was completely hydrolyzed by 10 cc. of 10% bean extract within half an hour by two varieties of soya bean, namely, Chiquita and Pekin, while only 44·4% of the urea was acted upon by the variety Tarheel; the other varieties of soya bean tested had a urease activity intermediate between these.

The urease activity of the beans examined showed no relation to the general appearance of the seeds; the size and shape of the seeds varied, as also did the colour of the seed coats.

An American Jack bean was tested, and found to be much more active than any of the varieties of soya bean, but a Jack bean grown locally in Sydney showed practically the same urease content as the more active varieties of soya bean.

The reaction at room temperature was not complete within five hours, although the Jack bean had completely hydrolyzed the urea present in the solution within thirty minutes at 51°C.

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THE ELECTROLYTIC PREPARATION OF CALOMEL FOR USE IN THE CALOMEL ELECTRODE

by

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The calomel electrode is universally used as a standard half-cell in conjunction with the hydrogen electrode for precise determinations of P_H, oxidation-reduction potentials, and such. For a strictly reproduceable standard half-cell, finely divided and very pure calomel is essential. In the method of preparation here outlined the calomel is made in the presence of N/10, N, or saturated KCl, as desired, the product is fine-grained and intimately mixed with mercury, and the process is less tedious and more easily handled than the usual electrolytic processes employed. In the usual methods of preparation (see, for example Hulett and Lipscomb (3) and Ewing (2)), the calomel is precipitated on a mercury surface, and continually wiped away with some stirring device. In the present method a mercury dropping electrode forms the anode, and the calomel is precipitated on the falling mercury stream, which carries down a fine layer of calomel, and at the same time continually presents a new mercury surface to the current. The arrangement can be followed from figure 1.

Into the vessel (A) is poured some pure mercury, and then a solution of KCl (normal, N/10, or saturated, according to the electrode required). B is a 400 cc. beaker, containing a saturated solution of cupric chloride, into which dips a copper electrode to form the cathode. A and B are connected by means of the bridge (C), which has a minimum diameter of 2 cm., this minimum being necessary to prevent any heating in the bridge. The lower extremities of the bridge are filled to a depth of 2 cm. with agar-gel, 2% agar in saturated KCl. The rest of the bridge is filled with a saturated solution of KCl. Controls showed that no copper nor hydroxyl ions diffused through the bridge during the process. The mercury dropping from the funnel (D) forms the anode, and has a platinum wire dipping into it, connected to the lighting circuit through an ammeter, and a suitable resistance.

Various methods of dropping were tried, in which the size and rate of the drops were varied. The most satisfactory results were obtained by allowing the mercury to flow through a piece of silk mesh (E) tied to the end of the funnel, whence it emerged as a very fine spray. As soon as the current is thrown on

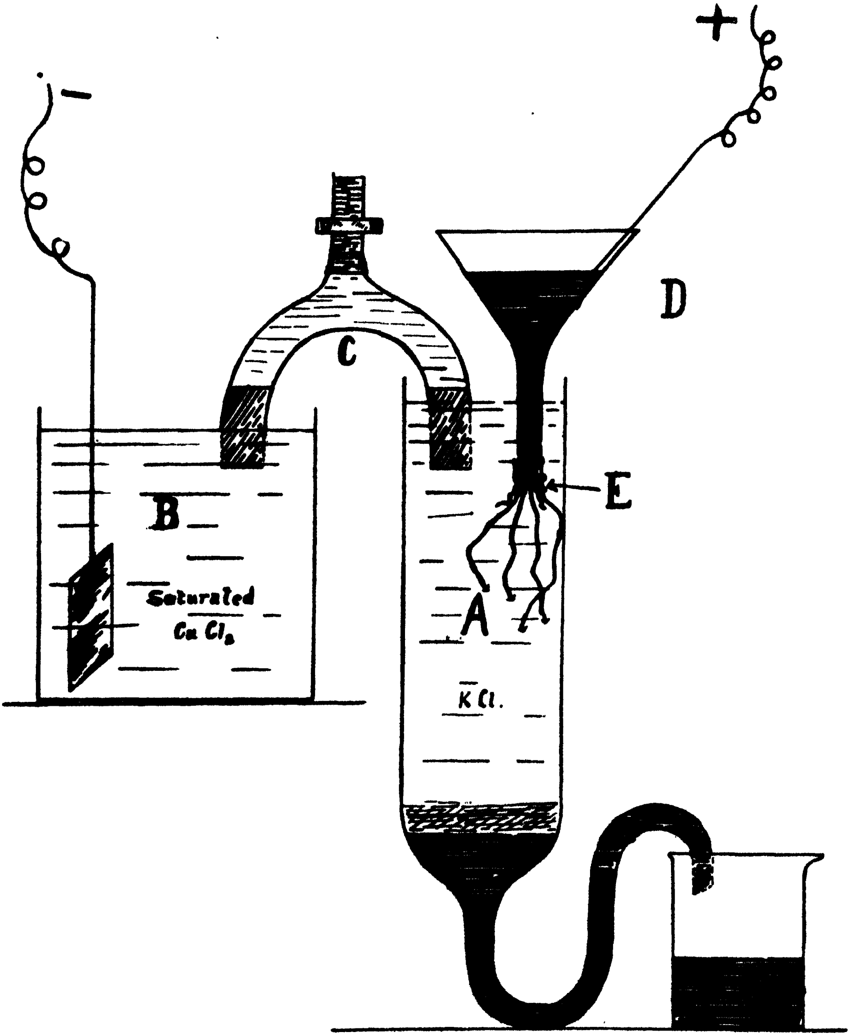

Figure 1.

the innumerable tiny drops coalesce into a dozen or so threads, as shown in figure 2. This effect is very striking and convenient, for these ever-changing threads continually present new surfaces to the solution whilst connected to the source of mercury supply and to the current. These threads have a maximum length, if the current is small, i.e., less than ¼ ampere, but in such cases the collected calomel is so intimately mixed with a relatively large bulk of mercury that it is difficult to get a calomel layer. It is preferable to use larger currents (1 to 2 amperes) when the emerging threads are much shorter, but the yield is much larger and more readily separated from the bulk of the mercury.

In this work the action was started with a small head of mercury in the funnel and a current of about 1½ amperes. As the action proceeded the mercury flow gradually fell off, due to a partial clogging of the silk, and then became fairly steady. At this stage the current was cut down to ¾ of an ampere, and the funnel filled with mercury, whence little further attention was necessary. A fine white sample of calomel, overlying a grey mass of finely divided mercury and calomel, was obtained, and two runs each of forty minutes yielded sufficient calomel for a dozen ordinary calomel electrodes. Eight such electrodes were made up, and gave strictly reproducible and correct values. Four of them were used in the chain below:

$$H_2,Pt,\ 0{\cdot}1N\text{–}HCl,\ sat.KCl,\ N\text{–}KCl,Hg_2Cl_2,\ Hg,$$

and they gave E.M.F. values of ·3483, ·3484, ·3483, and ·3483 volts.

During the above method of preparation the KCl in the anode chamber varies in strength. The collected calomel must therefore be well washed, and allowed to stand in contact with the standard solution of KCl of the required strength.

One advantage of this method is that we initially require pure samples of only two substances, KCl and mercury. The former can be purchased in the very pure state, but is generally recrystallized twice before use. The purest mercury can be recovered even from the most contaminated of laboratory stock in the following manner. The impure mercury is first run through a fine hole in a filter paper, and the gross impurities rejected. The sample is then well shaken with several lots of distilled water, and after the water is poured off the mercury is heated to boiling point in a beaker. It is then poured through a small funnel with a very long stem, the stem having been turned up for about 4 inches of its length and then turned down about the same distance, to end in a narrow orifice. The efficiency of this funnel in removing adhering contaminations is quite remarkable, and if the sample to be purified contains only such contaminations, and has not been used with substances that react with, or dissolve in, or form amalgams with, the mercury, then the process of purification can stop at this point. Various tests carried out in this laboratory have shown that in such cases this funnel delivers mercury which has all the properties of the pure distilled samples. If, however, the sample contains dissolved impurities, these are removed by spraying the mercury through a long column of 10% KOH solution, and then through a similar column of HNO_3 of density 1·1. Usually the mercury is sprayed from the fine point of a funnel, but a piece of silk tied over the end of a wide-stemmed funnel gives about a hundred times the efficiency. The mercury should be passed through these solutions at least twenty times. At this stage the mercury could be heated and dried, and then passed through the bent-stemmed funnel again, whence the product could be generally classified as very satisfactory, and suitable for all ordinary purposes. When the highest degree of purity is required, as, for example, in the preparation of the standard calomel cell, it is necessary to distil the purified sample, generally in vacuum. Of the various stills in use, the simplest to construct and to maintain is the self-pumping type described in Clark (1). Although it distills slowly, it has the advantage of requiring practically no attention. Such a still in this laboratory produces about 3 lb. of mercury per day, and the only attention it receives is the switching on of the current to the heating element in the morning and the switching off again at night.

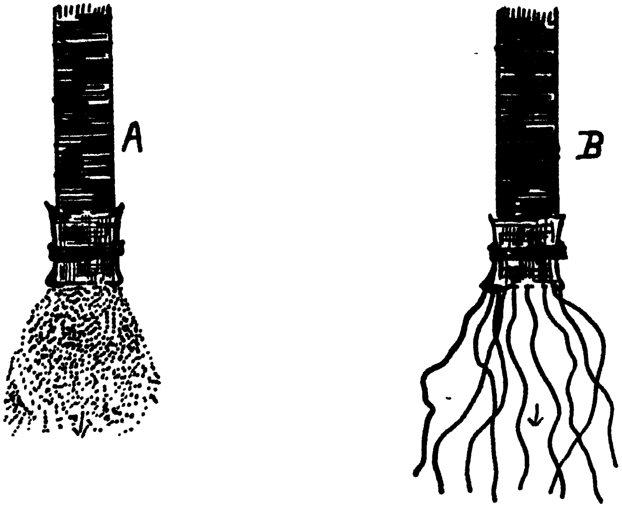

Figure 2.

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THE ACTION OF CERTAIN FAT-SOLVENTS ON ALCOHOLIC FERMENTATION

by

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The action of toluene on fermentation by yeast cells has been studied by Harden (2, 3), who found that the addition of toluene to a fermenting mixture of yeast cells and sugar causes a rapid fall in the rate of fermentation, until a relatively constant rate is attained much lower than that of the original mixture.

He further showed that the yeast acquires the property, not possessed by living yeast, of reacting to added phosphate in a manner similar to that of yeast-juice and dried yeast preparations. Thus in presence of toluene the addition of a soluble phosphate causes an increase in the rate of fermentation of sugar, which persists for a time, and then gradually falls practically to the original rate. During this period of enhanced fermentation the phosphate is converted into hexosephosphate, and Euler and Johansson (1) have used toluened yeast for the preparation of this substance.

Toluened yeast, however, reacts to phosphate less markedly than yeast-juice, but the property may be much increased by washing the yeast at the filter pump with toluene (3).

Another effect observed by Harden and Paine (4) is an increased autofermentation. The autofermentation of yeast is due to the fermentation of glucose produced from the glycogen of the cells by a diastatic enzyme, and as no increase in autofermentation results from the addition of toluene to yeast-juice, the effect on yeast cells cannot be attributed to direct action of the toluene on this enzyme. The action is considered by Harden as being probably due to the disorganization of the cell by the toluene, whereby the diastase finds more ready access to the glycogen.

He suggests that a similar action may also account for the action on the sugar fermentation, which may be associated with some special portion of the cell. Disorganization of the cell may produce altered conditions less favourable to fermentation, such as dilution.

On this hypothesis it may be assumed that when phosphate is added to yeast, although it is able to pass into the cells (6), it does not penetrate to that portion where fermentation occurs. In the cells altered by toluene it is able to come into contact with the fermenting complex, and so take part in the reaction as it does in the completely disorganized yeast-juice.

In the hope of throwing further light on the subject, experiments have been carried out on the action of toluene, and also of other substances having similar solvent properties, and like toluene being slightly soluble in water.

The samples of yeast employed were top yeasts, and were prepared by washing repeatedly in the centrifuge with water and pressing in a small hand-press.

The fermentations were carried out at 30°C., and the carbon-dioxide evolved measured at frequent intervals of time in the apparatus described by Harden, Thompson, and Young (5). The mixtures were previously saturated with carbon-dioxide at the temperature of the bath.

Effect on the Fermentation of Glucose.

In each case 10 grms. of pressed yeast were added to 20 cc. of water and 4 grms. of glucose, and 5 cc. of the liquid added. The following were tested: Toluene, benzene, xylene, ether, chloroform, ethyl acetate, amyl acetate, ethyl butyrate, butyl alcohol, and a sample of petroleum ether boiling below 60°C.

Of these an action similar to that of toluene was observed with benzene, xylene, ether, and ethyl butyrate. In each case the rate of fermentation rapidly fell, until a constant rate was attained. Examples are given in Table 1, with toluene, benzene, and ether, the figures referring to cc. of carbon-dioxide evolved in successive periods of five minutes. The experiments were done with different samples of yeast; 10 grms. of yeast in the presence of sugar usually gave from 40 to 50 cc. in five minutes.

TABLE 1.

Toluene.	Benzene.	Ether.
22·0	12·8	22·5
15·8	7·3	4·6
5·5	5·7	3·5
4·2	4·8	3·0
2·9	4·5	3·3
2·0	4·7	3·5
2·0	4·5	3·3

Complete cessation of fermentation was brought about by the addition of chloroform, ethyl acetate, amyl acetate, or butyl alcohol.

Petroleum ether reduced the rate of sugar fermentation, but not to the same extent as the other substances. In one experiment the addition of 5 cc. to 5 grms. of yeast in presence of sugar reduced the rate from 28 to 9·7 cc. in five minutes.

In another experiment yeast was stirred into about 100 cc. of petroleum ether, and after one hour filtered at the pump; 3 grms. of this gave a rate of 4·6 cc. in five minutes, as against 12·1 cc. for the untreated yeast.

Reaction to Phosphate.

For these experiments mixtures of 10 grms. of yeast, 20 cc. of 20% glucose, and 5 cc. of the liquid being tested, were incubated in the bath until the constant rate was attained; 5 cc. of a solution of sodium-phosphate ($Na_2HPO_4\ 12H_2O$) approximately 10%, and previously saturated in the bath with CO_2, were then added, and the rate observed every five minutes.

In most cases a typical reaction was obtained, the rate increasing to a maximum, and then rapidly falling to a constant rate, usually slightly higher than that of the original mixture. The additional carbon-dioxide produced during this period was obtained from the total fermentation and the final rate. The phosphate solutions employed were estimated by magnesium-citrate mixture, and the last column in the table gives CO_2 calculated from this (Na_2HPO_4 to CO_2).

The agreement was in general satisfactory.

A number of results are given in Table 2, the rates being given in cc. per five minutes.

TABLE 2.

Substance.	Initial Rate.	Maximum Rate.	Final Rate.	Extra CO_2 at N.T.P. Found.	Calctd.
Toluene, 1	3·3	9·2	4·8	40·4	43
„ 2	3·2	10·0	3·3	44·1	43
Benzene, 1	3·1	14·8	4·6	44·3	43
„ 2	2·8	11·1	3·0	44·1	43
Ether, 1	4·7	17·6	6·2	53·1	43
„ 2	2·6	14·0	3·9	49·0	43
Ethyl butyrate	3·0	12·5	3·4	40·1	43

Another series of experiments was carried out, in which the pressed yeast was stirred into about 200 cc. of the liquid, and allowed to stand for about fifteen minutes. It was then filtered at the pump and spread out on a sheet of filter paper, and allowed to stand for about an hour.

Weighed portions of this (usually 10 grms.) were fermented with 20 cc. of water, 4 grms. of glucose, and 5 cc. of phosphate added.

When treated with ether in this manner the yeast became very sticky, owing to plasmolysis, and in the later experiments 10 grms. of yeast were treated directly in the fermentation flask with the ether. After fifteen minutes the ether was drained off, and a current of air blown through the flask to remove as much ether as possible. The solutions were then added to the flask, and fermentation commenced.

TABLE 3.

Substance.	Initial Rate.	Maximum Rate.	Final Rate.	Extra CO_2 at N.T.P. Found.	Calctd.
Toluene, 1	3·6	9·5	3·7	41·2	42·3
„ 2	3·0	7·0	3·9	44·1	43·0
Benzene, 1	3·3	16·8	5·5	40·4	43·0
„ 2	3·1	14·8	4·5	41·0	43·0
Xylene, 1	5·1	8·3	5·2	40·6	43·0
„ 2	2·5	9·4	4·8	44·9	43·0
Ether, 1	6·2	26·2	6·7	45·1	43·0
„ 2	7·0	22·5	7·2	44·6	43·0

With ether, either when added to the mixture or when the yeast was treated beforehand with the reagent, there was a marked decrease in rate on the addition of the phosphate, and the maximum rate was only attained after some time. This slow increase to the maximum introduces an error into the calculation of the extra carbon-dioxide given off in the phosphate reaction. In one instance yeast, after treatment with ether, gave a rate with glucose of 6·8 cc. per 5 minutes. On addition of 5 cc. of phosphate the rate fell to 2·6 cc., and then gradually rose to a maximum of 27·8 after sixty minutes. It then rapidly fell to a final constant rate of 6·8 cc. The agreement between the calculated and found values for the CO_2 were therefore not so good in the ether experiments. In no case was a reaction to phosphate obtained with petroleum ether.

Table 4 shows that prolonged treatment with toluene did not destroy the activity of the yeast. Ten grms. of the treated yeast were used in each experiment, and 5 cc. of phosphate were added.

TABLE 4.

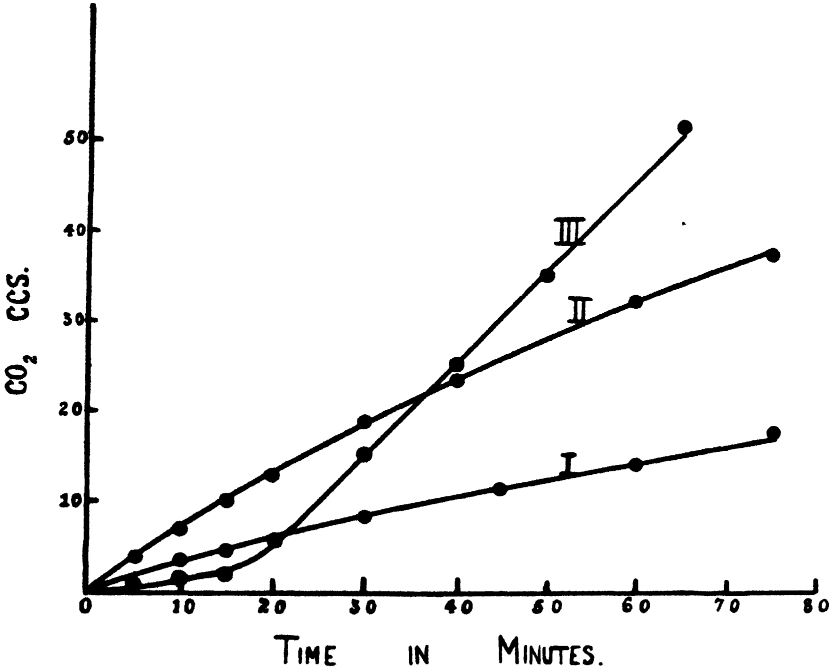
Time in Toluene.	Initial Rate.	Maximum Rate.	Final Rate.
15 minutes	3.6	9·5	3·7
30 minutes	3·8	10·8	3·9
15 hours	2·4	5·7	2·3

Effect on the Autofermentation.

The effect on the autofermentation was tried with petroleum ether, toluene, and ether, petroleum ether being tried because of its less marked action on the fermentation of sugar.

The Australian top yeasts employed showed a similar increase in the rate of autofermentation on the addition of toluene, to that described by Harden and Paine (4).

Ether exhibited the same effect, but in a more marked degree. In every case there was a period following the addition of ether, during which only a very feeble fermentation took place; then the rate increased, until a fairly constant rate was attained, which was higher than that given in presence of toluene.



These effects are seen in the accompanying curves, which refer to the following mixtures:

I. 10 grms. yeast + 25 cc. water.
II. 10 grms. yeast + 25 cc. water + 5 cc. toluene.
III. 10 grms. yeast + 25 cc. water + 5 cc. ether.

When petroleum ether was added to yeast and water the rate for the first few periods was usually high. This was probably due to the sample containing paraffins of low boiling point, which were vaporized at the temperature of the bath, especially when the flasks were shaken. After about thirty minutes, however, the rate became constant, and in the figures quoted the readings in the first hour have been discarded.

In one experiment the autofermentation of 10 grms. of yeast was 7·8 cc. in 40 minutes, whilst in the presence of 5 cc. of petroleum ether 43·6 cc. were obtained in the same time.

In another instance yeast was treated with petroleum ether for one hour, filtered at the pump, and the excess removed with a current of air; 5 grms. of this with 20 cc. of water gave a rate of 1·9 cc. per 5 minutes; 5 grms. untreated yeast gave 0·5 cc. per 5 minutes.

Effect on the Permeability of the Cell.

Yeast was washed with water in the centrifuge until the washings gave practically no reaction for phosphate. The yeast was then pressed. Two lots each of 10 grms. were treated with ether and toluene respectively by stirring with the liquids, standing for fifteen minutes, and then pouring off and draining.

To each was added 50 cc. of water, the mixtures shaken and allowed to stand for fifteen minutes, and centrifuged. The liquid was poured off, filtered, and the free phosphate estimated in 30 cc. of each, with magnesium-citrate mixture.

The following results were obtained:

TABLE 5.

	P_2O_5 in 50 cc. Filtrate.
10 grms. yeast untreated	0·0007 grms.
10 grms. yeast after toluene	0·0091 grms.
10 grms. yeast after ether	0·0381 grms.

In another series of experiments, lots of 10 grms. of yeast were treated as before with toluene and ether respectively. To each were added 20 cc. water, 4 grms. glucose, and 5 cc. of phosphate, the flasks were connected with the fermentation apparatus and incubated in the bath until the phosphate reaction had subsided. They were then centrifuged, and the free and total phosphate estimated in the supernatant liquids.

The figures obtained in three such experiments are given in the table, and represent P_2O_5 in 25 cc.:

TABLE 6.

		Free.	Total.
1.	After toluene	0·0011	0·0752 grms.
	After ether	0·0022	0·2165 grms.
2.	After toluene	—	0·1385 grms.
	After ether	—	0·1840 grms.
3.	After toluene	0·0052	0·0894 grms.
	After ether	0·0092	0·1488 grms.

These figures show that after treatment with either ether or toluene, both free and combined phosphates readily pass out of the yeast cells, but the permeability is more affected after ether than after toluene.

Further evidence that the yeast cell is more disorganized by ether than by toluene is furnished by the fact that after treatment with ether the coferment of alcoholic fermentation is readily washed out of the cell by water, whereas after toluene this does not happen.

Fifty grms. of pressed yeast were stirred up in a centrifuge vessel with 100 cc. ether, and allowed to stand, with occasional shaking, for fifteen minutes. The ether was then poured off, 100 cc. of water added, and shaken from time to time for about twenty minutes. It was then centrifuged, and the liquid decanted. The residue was further washed three times with water in the centrifuge.

Three lots of 10 grms. of the moist residue were weighed into flasks, and the fermentations measured in the presence of the following:

1. 2 grms. glucose + 25 cc. water.
2. 2 grms. glucose + 25 cc. first washings, after boiling.
3. 2 grms. glucose + 25 cc. water + 2·5 cc. phosphate.

The first gave no fermentation in twelve hours; the second showed an active fermentation, giving 77 cc. in the first hour.

The absence of fermentation in the third mixture showed that the loss of activity was not due to the washing out of phosphate.

In a second experiment, the yeast, after treatment with ether in a similar manner and subsequent washing, was pressed in the small hand-press. 10 grms. of this with water and sugar gave no fermentation in seventeen hours, whereas in the presence of the washings over 200 cc. were evolved in the same time.

Yeast treated with toluene and then repeatedly washed with water, was found to ferment glucose quite as actively in the presence of water as in that of the washings.

Treatment with ether, and subsequent washing with water, forms a very convenient method of preparing the alcoholic ferment free from coferment.

Preparation of the Hexosephosphates.

As the yeast cell after treatment with ether readily allows the hexosephosphates to pass out into the surrounding fluid, these substances can conveniently be prepared by this means. A weighed quantity of pressed yeast is put into a large flask, covered with ether, and well shaken. After about fifteen minutes the ether is drained off as much as possible, glucose solution is added, and the flask placed in a bath at 30°C., and phosphate solution added from time to time.

To control the addition of the phosphate, 10 grms. of the yeast are treated in an exactly similar manner, connected with the fermentation apparatus, and a corresponding amount of phosphate added. The rate of fermentation of this indicates when the reaction due to each addition of phosphate is completed, and the quantity which may be added. When the reaction is over the mixture is centrifuged, and the clear liquid employed for the preparation of the hexose diphosphate, as described by Young (8), or for both the monophosphate and the diphosphate, as described by Robison (7).

SUMMARY AND DISCUSSION.

1. Benzene, xylene, ether, and ethyl butyrate were found to have the same action on the fermentation of glucose by yeast cells as that already found by Harden for toluene, viz., a marked reduction in the rate.

Petroleum ether (below 60°C.) had a less marked effect; whilst chloroform, ethyl and amyl acetates, and butyl alcohol completely stopped the fermentation.

2. Benzene, xylene, ether, and ethyl butyrate, either when added to the mixture or when the yeast was previously treated with these liquids, caused the yeast to acquire the property of reacting to phosphate, with formation of hexosephosphate. This action was not found with petroleum ether.

3. Both ether and petroleum ether have a similar action to toluene in causing an increased rate of autofermentation, the effect of ether being the most marked.

4. When yeast was treated with ether or toluene the cells became more permeable to free phosphate and to hexosephosphates, and these substances could readily be washed out of the cells with water.

5. After washing yeast with ether, the coferment of alcoholic fermentation could be washed out of the cells with water. The yeast became inactive to glucose, and the fermentative activity could be restored by adding the washings to the mixture. This did not take place when toluene was used.

6. A method is given for preparing the two hexosephosphates with yeast which has been treated with ether.

The results suggest that yeast cells after treatment with petroleum ether, toluene, and ether represent three degrees of internal disorganization of the cells.

Petroleum ether, the least soluble in water of the three, alters the cell to the least extent. It increases the rate of autofermentation, decreases that of the sugar fermentation, but does not alter the cell sufficiently to give a reaction to phosphate.

Toluene has a greater action, so that it increases the rate of autofermentation, decreases that of sugar fermentation, the latter to a more marked degree, and the yeast is now able to react to phosphate. In addition it alters the cell wall so that phosphate and hexosephosphate may be washed out with water.

With ether the disorganization is more profound, so that in addition to those changes produced by toluene, the cell wall becomes permeable to the coferment, and this may be readily washed out with water.

Further evidence for this was given by the facts that all the cells were not killed by the treatment with petroleum ether, and cultures made into wort readily grew. On the other hand, in no case was any growth obtained when small pieces of yeast which had been treated with either toluene or ether were put into wort.

During the course of this work one of us (N.G.K.) has held a Research Scholarship of the University of Melbourne.

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THE ACTION OF ETHER ON THE YEAST CELL
I. CARBOXYLASE

by

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(From the Biochemical Laboratory of the University of Melbourne).

(Submitted for publication 13th August, 1926.)

In the previous communication (2) it was shown that living yeast which has been treated with ether is altered in such a manner that the coenzyme of alcoholic fermentation may be readily removed by washing with water. The residue is quite inactive to glucose, and requires the addition of the washings in order that fermentation of sugar shall take place. It behaves, therefore, exactly like zymin (acetoned yeast) and other dried preparations of yeast.

It was shown by Harden (1) that such preparations were able to transform pyruvic acid into carbon-dioxide and acetaldehyde, even after they had been rendered incapable of fermenting glucose by removal of the coenzyme with water. As this action of carboxylase on pyruvic acid is generally considered to be one of the reactions in the change from glucose to carbon-dioxide and alcohol, it is clear that the coenzyme is not concerned with this stage in the fermentation of glucose (see also Neuberg and Gottschalk, 3).

The experiments described below showed that yeast which had been treated with ether, and the coenzyme subsequently removed with water, behaved in a similar manner to washed zymin.

In each case 50 grms. of top yeast were shaken up with about 100 cc. of ether, and after standing for fifteen minutes the ether was drained off as much as possible. The yeast was then shaken with 100 cc. of water, allowed to stand for thirty minutes, and the mixture centrifuged. The residue was further washed with water four times, finally suspended in 100 cc. of water, and this mixture used for the fermentations.

The first washings were boiled to remove the ether, and employed as coenzyme solution.

One experiment was tried with 0·25 gr. of pyruvic acid added to 25 cc. of the yeast suspension, but the mixture was too acid, and only a very slight action was obtained. The method used by Harden (1) was therefore employed, in which the pyruvic acid was neutralized with alkali, and the fermentation carried out in the presence of boric acid to decompose any alkali carbonate formed.

The following results were obtained:

Experiment 1. Date, 30/6/26.

1. 20 cc. yeast suspension + 25 cc. 1% pyruvic acid + 2·5 cc. N.NaOH + 3 gr. boric acid gave 29·2 cc. CO_2 in 75 minutes.
2. 20 cc. yeast suspension + 25 cc. water + 2 gr. glucose gave no fermentation.
3. 20 cc. yeast suspension + 25 cc. coenzyme + 2 gr. glucose gave 32·5 cc. CO_2 in 75 minutes.

Experiment 2. Date 7/7/26.

1. 25 cc. yeast suspension + 25 cc. 1% pyruvic acid + 2·5 cc. N.NaOH gave 18 cc. CO_2 in 50 minutes.
2. 25 cc. yeast suspension + 25 cc. water + 2 gr. glucose gave 0·8 cc. CO_2 in 50 minutes.
3. 25 cc. yeast suspension + 25 cc. water coenzyme + 2 gr. glucose gave 28·2 cc. CO_2 in 50 minutes.

In both experiments the formation of acetaldehyde was shown by the smell, and by the production of a colour with Schiff's reagent.

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A CONVENIENT HYDROGEN ELECTRODE

by

J. M. LEWIS

(From the Physiology Laboratory, University of Melbourne).

(Submitted for publication 14th August, 1926.)

The electrode about to be described was evolved during the course of some work on which the writer was recently engaged. The simplicity of its construction and the ease with which it may be used, where the hydrogen atmosphere employed is stationary, were such that it seemed worth while to enter into its description for the benefit of others to whom it might prove useful. As will be seen, the electrode tube may be regarded as a modification of the well-known form devised by Michaelis.

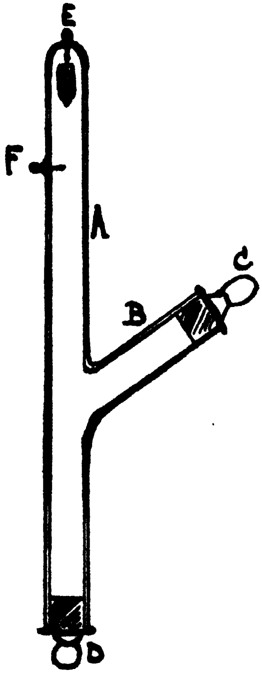


Figure 1.

The Michaelis electrode depends for the completion of the chain between the hydrogen and calomel electrodes upon a tape or wick moistened with saturated potassium-chloride solution. Such wicks or tapes are often inconvenient to use, and it occurred to the writer that as it is possible to work with the tap of the Barendrecht calomel electrode closed, provided that it be not greased, it should be possible to design a hydrogen electrode which would complete the chain through the fluid present round an ungreased glass stopper.

As a matter of fact, there is sufficient capillary transport about the stopper in the Barendrecht calomel electrode when closed to permit the solution in the vessel to escape in slowly forming drops. This proves useful in preventing the diffusion of contaminating solutions back into the calomel electrode during use, and is thus of the greatest service.

The author's form of electrode consists of a vertical tube (A, figure 1), about 9 cm. in length and 1 cm. in diameter. To this, at about 3 cm. from its lower end, is joined a side tube (B), making an angle of about 45° with the main tube. Both the main and side tubes are closed by stoppers. The difficulty caused by having to grind in these stoppers may be avoided by cutting off the necks of suitably sized flasks, having stoppers already fitted to them, and fusing these to the electrode tubes.

At the upper end of the main tube (A) a platinum electrode (E), formed from heavy foil, is fused in position, and some little distance below this a piece of stout platinum wire (F) is also fused in. This latter is made to serve as the anode during the deposition of platinum black on the electrode proper. The exact form of the electrode (E) is not important, but it may be rolled slightly upon itself, and is shown in the illustration as having a pointed free end. This pointed end may be used to obtain "minimum contact" with the contained solution if so desired.

A convenient stand is shown in figure 2. In this the electrode tube is clipped by a spring in a V-shaped groove made in a block of brass, which in turn is freely adjustable as to height on a light steel rod. This standard is housed on a heavy foot, which is hollowed out as shown for the accommodation of a small beaker.

After the electrode is constructed it must be carefully cleaned by the usual methods to render it free from chemical impurities and grease, after which it will be ready for platinizing. The presence of the platinum wire (F, figure 1) renders the operation of platinization very simple, and the fixation of the electrode within the tube does away with the danger of contamination, which is always present when the platinum foil or wire has to be removed from the tube for this purpose.

The platinizing of the foil is effected by removing the stoppers from both main and side tubes, inverting the apparatus, and running in the platinizing solution until the wire (F) is completely immersed. The tube is now placed in its stand in the inverted position, E and F being connected to the negative and

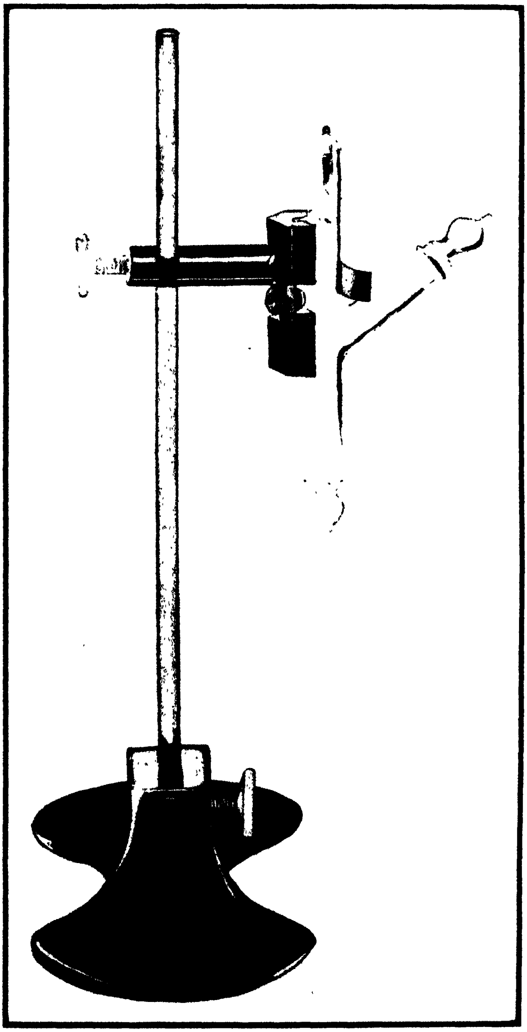


Figure 2.

positive terminals respectively of an accumulator, the coating and subsequent treatment of the electrode being carried out in the usual manner.

In measuring the hydrogen ion concentration of an unknown solution the stopper (C) is placed in position, and the solution is poured in at D until it is within a short distance of the opening. The stopper (D) is now inserted, and the tube is brought into the horizontal position with the side tube (B) uppermost. This allows the entrapped air to be caught in the tube (B). The electrode is now brought into the vertical position, with the electrode (E) uppermost, and the stopper (C) is removed. By means of a hollow platinum needle, such as is used in surgery, fused into a glass tube, hydrogen is bubbled into A. The gas displaces the liquid, which tends to fill the side tube (B). When the level of the fluid in A has fallen sufficiently for the end of the electrode (E) to be just covered, the platinum needle is withdrawn, and the side tube (B) is completely filled with the solution. Using a rotary motion, the stopper (C) must be so inserted that no bubble of air is trapped in the tube. The apparatus is now dried with a cloth, and is vigorously shaken with the hand for a few minutes, after which the stopper (C) is removed, and that at D is cautiously moved, so that it is completely wetted with the contained solution, and is kept in position by just enough pressure to prevent its dropping out. The tube is now clipped in position on its stand, and the carrier is lowered so that the stopper and end of the tube at D is well covered with a saturated solution of potassium-chloride contained in a small beaker. A fine wire from the negative terminal of the potentiometer is carefully hooked into the platinum loop at E, and on dipping the tube from the calomel electrode also into the small beaker below D, the chain is complete and a measurement may be made.

With this electrode a reading may be made which is sharp enough for most purposes, though, as would be expected, the results are more satisfactory when one is using the apparatus with solution of good electrical conductivity.

CORRECTION.

The following correction should be made on p. 130 to
The Milk of Australian Women
by
H. S. Halcro-Wardlaw and Ellice E. P. Dart.

Page 130, line 17, delete the word "not"; line 18, delete the word "minimum" and the words following "hours"; line 19, delete this line.

STUDIES IN TRYPTOPHANE FEEDING I.

by

C. S. HICKS

(From the Biochemical Laboratories, Cambridge, England).

(Submitted for publication 8th October, 1926.)

The Kendall formula for thyroxin, and the more recent work of Wier (1) on the tryptophane content of thyroid gland, suggested that the amino-acid might be the parent substance of the essential secretion. Animal experiments were devised at the instigation of Sir F. G. Hopkins to determine:

(*a*) What changes, if any, occur in the thyroid tissues of an animal on a diet deficient in tryptophane.

(*b*) What changes might similarly occur in heat output.

The first diet chosen was the original Hopkins acid hydrolyzed casein mixture (2).

[1]Amino acid from acid hydrolysis of casein	= 23%
Starch	= 40%
Sugar	= 17%
Fat	= 15%
Salt	= 5%
Cystine	= 1%

[1]Tested for absence of tryptophane.

The animals, which were of uniform stock from Mottram and Hartwell's breeders (3), were divided into the following groups:

(1) Six controls on corn, bread and milk, and vegetables.

(2) Six controls on synthetic diet + tryptophane in amount equal to 2% of total protein fraction.

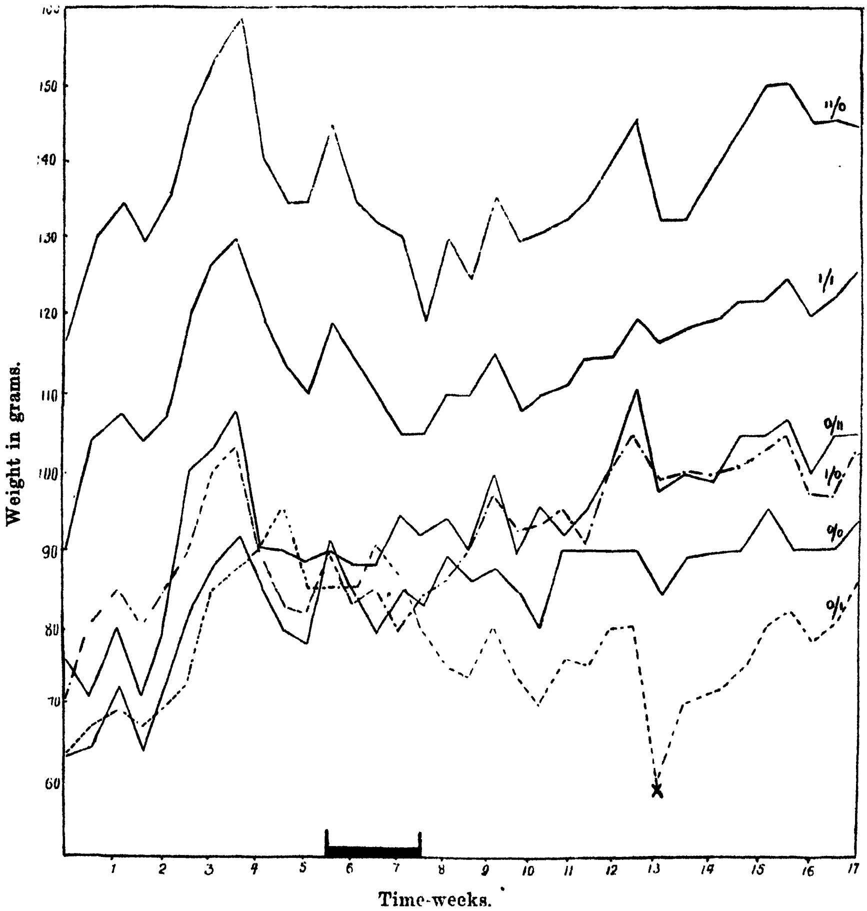
(3) Four rats on synthetic diet containing 0·5% tryptophane.

(4) Six rats on synthetic diet free from tryptophane.

As controls on the specific nature of the deficiency, two rats from Experiment 4 were removed midway through the experimental period and placed on diet (3). Vitamins were supplied in clarified butter, alcohol-cleared marmite extract, and filtered orange juice.

The tryptophane was extracted after preparation by Dakin's butyl-alcohol method, and was free from traces of heavy metals; 80 grms. were prepared.

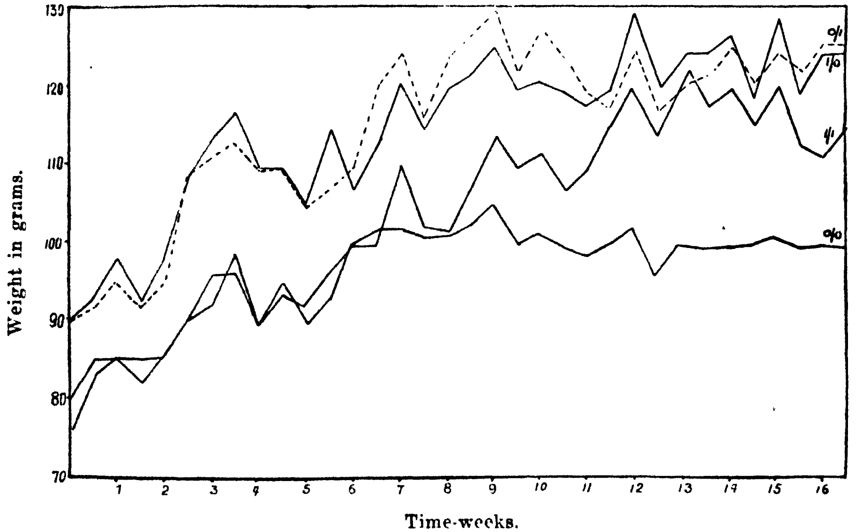
The animals were watched very closely, and were housed in roomy cages, each 2 ft. 6 in. x 2 ft. x 2 ft. in dimensions, and fitted with exercising wheels. The rats were entirely free from vermin, catarrh, or skin disease, and scrupulous hygiene was maintained.



Growth-curves of rats from Series (2) on diet containing 2% tryptophane in the amino-acid fraction of the mixture. The shaded area represents the period during which the tryptophane was increased in amount to 3%, and it was maintained at that figure for Rat 1/0 until the point X.

It was found that within four weeks the synthetic diet was being almost entirely rejected, and that voluntary starvation, in whole or part, was playing a greater part than any deficiency in diet could do. The starch was reduced to 30% and the sugar raised to 27% to make a more palatable diet, and there followed a temporary revival of appetite, which, however, soon failed again. Only by altering the manner in which the food was presented, e.g., in gelatin jelly variously flavoured, etc., could a full intake of food be assured.

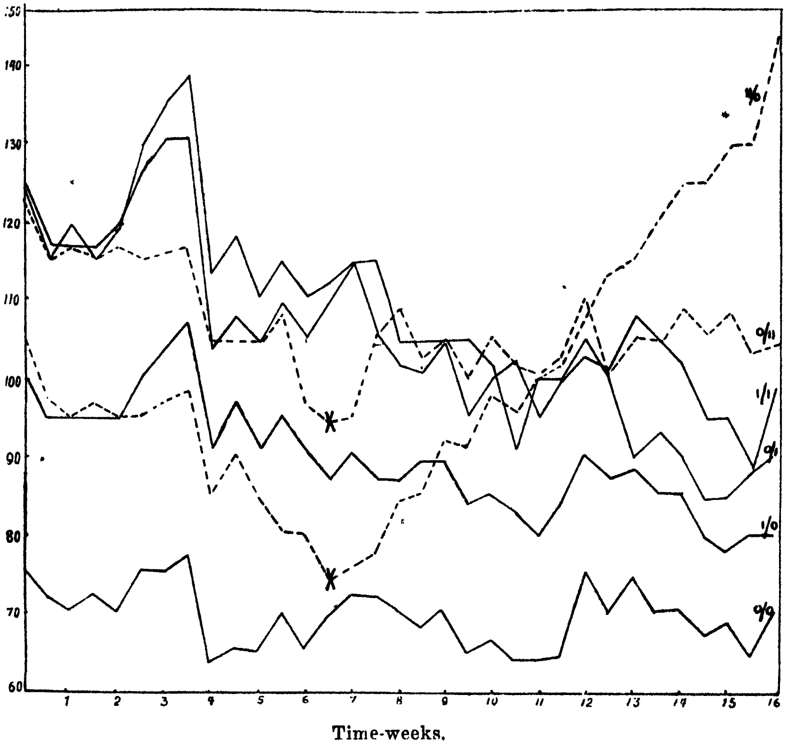
The other experimental sets receiving added tryptophane, although evincing some distaste for the food, never completely rejected it, and careful preparation readily corrected any slackening in intake. This was attained by subjecting all food to the same manipulation as in the case of Experiment (4) in order to maintain uniformity throughout. From the curves it will be noted that the growth failure is not so striking as was anticipated, and this may be due to the great care taken to ensure a nearly normal food intake, cf. Hopkins (2).



Growth-curves of animals from Series (3) on diet containing 0.5% tryptophane in the amino-acid fraction of the mixture.

Two of the most debilitated animals were taken from the series and resuscitated by diet (3), great pains being taken to ensure their recovery by making the food as palatable as possible; unless this had been done these animals would certainly have succumbed. The fur was "glairy," the muscles limp, and the feet blue and numbed-looking. There was some incontinence, and the urine stained the fur an orange-yellow colour. The rectal temperature disclosed no deviation from normal, which, in the rat, is subject to a rather wide range of fluctuation.

At no time, however, was the tendency to spontaneous exercise absent, even though the tendency to huddle together, as though cold, was noted. The recovery was so complete and surprising as to leave no doubt as to the absence of tryptophane being the cause of their previous failure in growth and maintenance. It was found that the series receiving 0·5% tryptophane in the amino-acid moiety of the diet did much better than those on 2%, their appearance more than their growth-curves illustrating this fact. When the amount of tryptophane was raised to 3% of the amino-acid fraction, these animals became markedly worse,

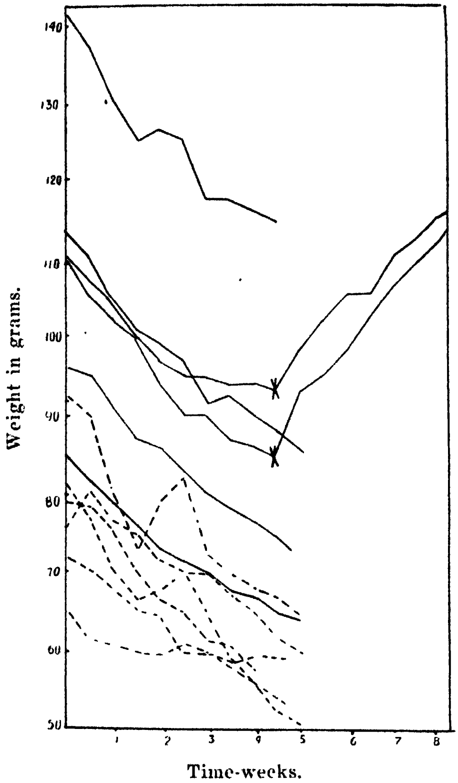


Growth-curves for animals from Series (4) on diet free from tryptophane. The dotted curves represent the growth of two animals to whose diet tryptophane was restored at X.

and resumed growth only on resumption of a 0·5% tryptophane diet. These animals looked quite like those in the early stages of feeding on a tryptophane-free diet. At post-mortem the animals on tryptophane-free diet showed complete absence of subcutaneous fat, although abdominal fat appeared normal in amount. The skin was parchment-like, and covered with sparse, matted hair, the rats resuscitated by restoration of tryptophane to the diet showing normal subcutaneous fat and a pliable skin covered with thick fur.

The histological changes observed in the thyroid glands will be discussed in a later paper.

Heat production was studied after the night's fast by the method of Prince (4), the animals being given a small dose of luminal sodium prior to the test (5).



Growth curves from series of rats on Zein diet. Tryptophane was restored in two cases at X. Dotted curves are for females. All animals were moribund by the end of the fifth week.

The latter was made up in sterile saline, 0·08 grm. per cc. Dose, ·1 cc. per 200 grm. rat. This was enough to induce sleep if the animal was kept in the dark and undisturbed for some 15 to 20 minutes.[2] Results are given in terms of calories per unit surface area, calculated by the method of Krogh (5):

$$\text{Calories per kilogram} \times \sqrt[3]{\text{Weight in kilos}}.$$

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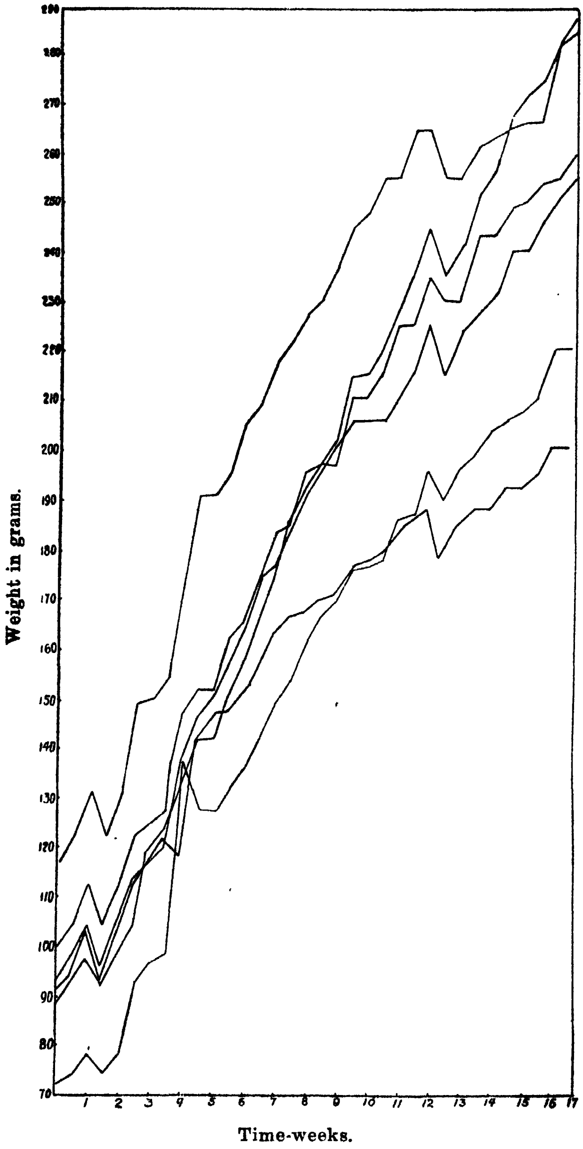
[2] It is to be especially noted that the animals used were handled daily. They were exceedingly tame, and submitted to experimentation with no difficulty whatever.



Curves of growth of control Series (1). Diet of corn, bread and milk, and fresh carrots. All growth-curves are drawn to same scale, and show the great difference between normal and synthetic food "controls" over same period.

In the case of the second series of respiratory exchange determinations on rats of a Zein diet, the greatest care was taken to ensure standard conditions, owing to the results of the first series showing so small a deviation from normal. In order to illustrate the experiments, full numerical details are quoted for analysis. The unsatisfactory nature of the hydrolyzed casein diet led to a repetition of the work, using zein as protein supply, the diet being constituted as follows (6):

Zein	16·92%	
	·54%	Lysine hydrochloride
	·27%	Cystine
	·26%	Na_2CO_3
	26·34%	Potato starch
	5·00%	Salt mixture
	23·13%	Sucrose
	15·00%	Butter
	12·00%	Lard
	½ cc.	Marmite extract

The growth-curves show a marked difference from the previous set. The failure to maintain weight is more marked, and despite the fact that no difficulty was experienced in ensuring that the diet was eaten, the duration of the experiment was reduced to one-third of that of the previous set. Using Tigerstedt's (7) figure of 227 calories per kilo of rat per 24 hours as a basal heat output, Krogh calculates the heat per unit of surface as 111. It will be seen that the basal heat output falls below normal in the case of the animals on tryptophane-free diet, but those receiving tryptophane are only relatively better in this respect, the figures giving no real indication of any specific effect. This could be due to the tissues supplying the needed amino-acid at their own expense, until death terminates the experiment, but it might be expected that some indication would be obtained, especially in the later stages. It might be pointed out that the rate of growth of the animals to whom tryptophane was restored is greater than those on full tryptophane diet throughout. The respiratory quotient and heat output follow the growth in this respect. The respiratory quotient of the animals on tryptophane-free diet points to metabolism of fat, or possibly, in the case of the lower figures, formation of carbohydrate from protein (8).

SUMMARY OF CONCLUSIONS.

1. Absence of tryptophane from a diet causes a falling off in heat production commensurate only with starvation wasting. There is no evidence from measurement of respiratory exchange that it is required for the elaboration of the thyroid hormone.

2. There is no fall in body temperature, even in the extreme stages of tryptophane-deficient feeding.

3. There is evidence that added tryptophane in a synthetic diet differs in its action from that combined in the protein molecule. It may cause toxic symptoms when increased beyond 2% of the protein fraction. The minimum amount of tryptophane necessary as such in a synthetic diet appears to be in the vicinity of 0·5% of the protein fraction. The optimal amount would appear to lie near the same figure.

4. Zein provides a better basis for such experimentation, the results of which, unless closely watched, are vitiated by voluntary starvation, particularly when hydrolyzed casein forms the basis of the diet.

From the results of the above long and carefully controlled experiments the latter would appear to be an unsatisfactory source of amino-acid, a fact not unconnected with the destructive action of the treatment upon the diketopiperazine orientations pre-existing in the protein molecule.

Thanks are due to Professor Sir F. G. Hopkins for his advice throughout this work, which was carried out for the Medical Research Council during the tenure of a Beit Fellowship for Medical Research.

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TABLE 1.

Animals on Hydrolyzed Casein Synthetic Diet with Added Tryptophane.

Series (2).

Cage 4.

Date	Marks	Rectal Temp.	R.Q.	Weight Grms.	C x $\sqrt[3]{W}$ Per 24 hrs. *C = Calories per Kilo per 24 hrs. W=Weight in Kilos.*
9.11.25	0/1	—	0·78	95	84·0
9.11.25	11/0	—	0·68	152	79·2
11.11.25	11/0	—	0·76	148	76·8

Date	Marks	Rectal Temp.	R.Q.	Weight Grms.	$C \times \sqrt[3]{W}$ Per 24 hrs. *C = Calories per Kilo per 24 hrs. W = Weight in Kilos.*
13.11.25	0/0	—	0·74	92	72·0
13.11.25	1/0	—	0·72	97	84·0
29. 4.25	11/0	96·7	0·68	154	86·0
29. 4.25	1/1	96·4	0·69	135	86·4
30. 4.25	0/0	95·0	0·70	105	79·2
30. 4.25	1/0	95·5	0·71	112	74·4
30. 4.25	0/1	94·8	0·73	90	76·8
30. 4.25	0/11	95·0	0·71	115	76·8

TABLE 2.

Animals on Zein Diet free from Tryptophane.

Cage 3.

Date	Marks	Rectal Temp.	Weight Grms.	R.Q.	$C \times \sqrt[3]{W}$ Per 24 hrs. *C = Calories per Kilo per 24 hrs. W = Weight in Kilos.*
8.11.25	0/0	—	60	0·78	79·2
8.11.25	1/0	—	85	0·78	76·8
8.11.25	11/0	—	105	0·70	74·4
17.11.25	1/1	—	102	0·68	72·0
17.11.25	0/1	—	106	0·68	81·6
17.11.25	1/0	—	87	0·67	74·4
24.11.25	1/0	97·0	80	0·63	72·0
24.11.25	0/1	97·7	107	0·72	86·0
10. 3.25	0/1	95·6	100	0·68	74·4
10. 3.25	0/0	97·8	65	0·66	74·4
18. 4.25	1/1	97·4	95	0·69	76·8
18. 4.25	1/0	97·4	85	0·70	69·6
21. 4.25	1/0	97·4	78	0·68	62·4
21. 4.25	0/1	100·0	80	0·65	67·2

TABLE 3.

Experimental and Derived Data.

Tryptophane-free Zein Diet.

Time	Marks		Weight, Grams	Barometer	Total Volume Air Collected, cc.	Duration of Experiment	CO_2 %	O_2 %	CO_2 per Minute	O_2 per Minute	R.Q.	Air Temp., °C.	Calories per Kilo. per 24 hrs. = C.	$C \times \sqrt[3]{W}$ (W = Kilos) (24 hrs.)	Date and Cage No.	
12.0	None	♂	94	757·3	6543	35	0·960	1·33	1·645	2·284	·72	22·9	164	74·4	27.11.25	K
11.15	0/11	♂	100	777·5	6000	40	1·140	1·58	1·432	1·961	·73	22·2	132	61·3	5.12.25	K
12.15	0/1	♂	118	775·5	6622	35	1·160	1·80	2·076	3·209	·65	21·9	165	81·0	5.12.25	K
4.0	1/1	♂	95	760·5	6008	35	1·187	1·79	1·830	2·860	·63	20·2	202·8	92·5	7.12.25	K
4.0	0/0	♂	86	752·6	6780	40	1·225	1·89	1·860	2·880	·647	20·85	175·2	77·3	8.12.25	K
12.30	1/1	♂	101	755·5	7231	45	1·373	1·06	1·310	1·970	·77	21·8	131·5	61·2	9.12.25	K
3.30	11/0	♂	80	755·3	5881	45	1·660	2·29	1·990	2·750	·72	21·6	185·0	86·0	10.12.25	K
12.0	11/0	♀	68	758	5878	48	1·064	1·48	1·303	1·812	·718	23·0	179·6	73·3	11.12.25	L
11.30	1/0	♀	68	772·2	6527	40	0·920	1·22	1·500	1·990	·750	23·6	197·3	80·5	16.12.25	L
11.30	0/1	♀	90	771	6370	46	1·580	2·03	1·940	2·53	·76	23·7	189·5	84·9	17.12.25	L
11.30	0/11	♀	58	765	5900	40	1·020	1·62	1·390	2·21	·63	23·6	255·0	98·9	18.12.25	L

Mean error for series $C \times \sqrt[3]{W} = \pm 10{\cdot}7$

CAN THE CATTLE TICK HAEMAPHYSALIS BISPINOSA ACT AS THE CARRIER OF PIROPLASMOSIS (*PIROPLASMA BIGEMINUM*)?

AN EXPERIMENTAL ENQUIRY

by

JOHN LEGG

(Government Veterinary Surgeon, Townsville, Queensland).

(*Submitted for publication 18th October, 1926.*)

The work, of which an account is given below, was undertaken at the request of the New Zealand Department of Agriculture, its object being to prove whether the cattle tick, *Haemaphysalis bispinosa*, which is widespread over part of the North Island of New Zealand, is capable of acting as the intermediary in the transmission of *Piroplasma bigeminum*, the causal organism of piroplasmosis of Queensland cattle.

So far as is known, *Haemaphysalis bispinosa* does not act as a vector in the spread of any known contagious disease of cattle, or for that matter of any other domesticated animal, on which it may be found in one or other of the stages of its life history. In this respect is differs from many other members of the Ixodoidea, and its only effect, though serious enough, is that of an external parasite.

In Queensland, on the other hand, is found *Margaropus annulatus* var. *australis*, another species of cattle tick, which is not only in itself a serious external parasite of cattle, but one that acts as the intermediary for *Piroplasma bigeminum*.

In years gone by, in Queensland the tick *Margaropus australis* gradually spread from the northern towards the southern coastal areas of the State; serious outbreaks of piroplasmosis occurred, causing a mortality as high as 95 per cent. amongst the highly susceptible cattle, and the effects of the tick as an external parasite at that time were relatively negligible. At the present moment outbreaks of piroplasmosis which occur in Queensland are practically wholly confined to areas of country until recently clean, into which the tick has made an incursion, or where cattle are removed without vaccination from clean into tick-infested areas.

The reason for the occurrence of so few serious outbreaks of piroplasmosis amongst cattle in the tick-infested areas of Queensland is the well-known one, viz., that calves soon after birth become infected with the piroplasm, and being highly resistant, develop early in life a strong immunity, or rather a tolerance to the disease, which toleration is being continually reinforced throughout the life of the animal through continual reinfection with ticks.

That the piroplasm exists in greater or lesser numbers in the blood of such animals is shown, firstly, by the fact that when the blood of such animals is inoculated, either subcutaneously or intravenously, into other susceptible animals, a reaction is frequently, though not always produced, with the appearance of piroplasms in the circulation of the latter. Secondly, we know that an animal which has recovered from piroplasmosis may suffer at some future date from another disease, and this latter may so lower the resistance of the animal that an increase of the piroplasms within the circulation follows, and piroplasmosis results. Such is a common experience with cattle reared in tick-infested areas in Queensland.

All Queensland cattle bred on tick-infested country, therefore, may be regarded as being tolerant to piroplasmosis, owing to infection when young, and the piroplasms may be regarded as being present in greater or lesser numbers in the circulation.

In New Zealand, on the other hand, the cattle tick, *Haemaphysalis bispinosa*, exists over a fairly wide area, but, as before stated, it does not act like *Margaropus annulatus* as the agent in the transmission of any known disease. This may be due, however, not to its inability to do so, but to the fact that the first of such ticks to enter New Zealand, and whose progeny is represented by the ticks at present there, may have been themselves "clean."

As New Zealand and Australia are countries not widely separated from each other, the question arises as to whether cattle which had spent some period of their lives in areas infected with *Margaropus annulatus*, and were thus infected with *Piroplasma bigeminum*, could with safety be transported to New Zealand, and into the areas infected with *Haemaphysalis bispinosa*, without setting up an outbreak of piroplasmosis. In other words, could *Haemaphysalis bispinosa* convey piroplasmosis, or redwater, as the disease is commonly called, in the same manner as *Margaropus annulatus?*

Comparison of the Life History of Margaropus annulatus with that of Haemaphysalis bispinosa.

A comparison of the life histories of these two ticks shows that one, *Margaropus annulatus,* is a continuous, and the other, *Haemaphysalis bispinosa,* is an interrupted feeder; in other words, one is a single host tick, and the other a three host.

The life history of the *Piroplasma bigeminum* in its stages through the tick, *Margaropus annulatus*, has not been worked out, but we know that the larvae raised from adults which have engorged on an infected or recovered animal are themselves infective. Neither has the biology of the tick, *Margaropus annulatus*, been fully worked out in Queensland, but so far as is known, the young larval ticks first moult about seven days after attaching to their host, and the second moult, from the nymph to the adult, takes place about the sixteenth day. These periods will possibly be influenced somewhat by the seasonal conditions prevailing at the time. Thus it has been found in America that the tick, *Margaropus annulatus*, may remain on its host up to 58 days, although the usual period, at least during the warmer months of the year, is 21 to 28 days. When the female *Margaropus annulatus* drops off, she may be found ovipositing within 4 days, and these eggs may hatch within 21 days (minimum), so that this tick would take at least 46 days to complete its life history from larvae to larvae. Under natural conditions it would probably be much longer.

Haemaphysalis bispinosa, on the other hand, is an interrupted feeder. It has been found under Queensland conditions to take at least 4 days to engorge and drop off, in each of the various stages of its life history, i.e., larvae, nymph, and adult, and at least 10 days to complete each of the two moults, so that to complete the period from unengorged larvae to engorged adult is at least 32 days. No figures are available to show the preoviposition period or the period of incubation of the eggs under Queensland conditions, but they are certainly not less than those of *Margaropus annulatus*, viz., 4 days and 21 days respectively. Thus it will be seen that even under artificial conditions the life cycle of *Haemaphysalis bispinosa* takes somewhat longer to complete than that of *Margaropus annulatus*. Under natural conditions, where both the newly-hatched nymph and the adult have to await their chances on the grass of attaching themselves to a passing beast, a circumstance which does not apply to a continuous feeder like *Margaropus annulatus*, it will be easy to recognize that *Haemaphysalis bispinosa* takes much longer to complete its life history than *Margaropus annulatus*.

Turning now to the question of the possibility of *Haemaphysalis bispinosa* acting as a transmitter of *Piroplasma bigeminum*, it will be noted, that being an interrupted feeder, it will be necessary to test the capacity of this tick to transmit the disease in any of the three stages of its life history by "infecting" the tick in the immediately prior stage. Even then, if the result were negative, there would still remain the possibility that the tick could convey the disease in a further subsequent stage. These latter possibilities are not likely with *Piroplasma bigeminum*, although we know that heartwater, as shown by Lounsbury, can be conveyed by the interrupted feeder, *Amblyomma hebraeum*, in the adult stage when it has fed on an infected animal in the previous larval stage, and on a non-infected or non-susceptible animal in the nymphal stage.

Keeping these possibilities in mind, a series of experiments were therefore outlined, in which the tick, *Haemaphysalis bispinosa*, was "infected" in each of the three stages (larvae, nymph, and adult), and then tested on non-immune animals in each of the three subsequent stages of its life history.

The experiments might be set out as follows:

"A" Series, where the ticks were engorged on an immune or recovered animal in the larval stage, then after moulting fed as nymphs on a non-immune animal (Experiment 1).

Young adults raised from the engorged nymphs from the last experiment were then tested on a second non-immune animal (Experiment 2).

From the engorged adults obtained from Experiment 2, larvae were raised, and these were allowed to engorge on a third non-immune animal (Experiment 3).

"B" Series, where the ticks were engorged on an immune or recovered animal in the nymphal stage, and then after moulting, fed as adults (Experiment 4) on a non-immune animal. From the engorged adults obtained from this experiment larvae were raised, and these were engorged on a second non-immune animal (Experiment 5).

From these engorged larvae nymphs were raised, and these were tested on a third non-immune animal (Experiment 6).

"C" Series, where the ticks were engorged in the adult stage on an immune or recovered animal, then after ovipositing and producing larvae, these were fed on a non-immune animal (Experiment 7).

From the engorged larvae obtained in the last experiment nymphs were raised, and these were engorged on a second non-immune animal (Experiment 8).

From the engorged nymphs obtained in the last experiment adults were raised, and these were engorged on a third non-immune animal (Experiment 9).

Details of Experiments.

In carrying out these experiments both immune and non-immune cattle were required. The former are common enough in the Townsville district, and as the tick is found for a distance of 200 miles to the west, it was found necessary to obtain the latter outside this area for the purpose of experiment.

In regard to the use of an immune animal bred on country infested with *Margaropus annulatus*, and using this animal for the purpose of "infecting" the ticks, *Haemaphysalis bispinosa*, it has been found that the citrated blood of many such animals, taken indiscriminately, frequently fails to invoke a reaction, even when injected in large doses into cattle highly susceptible to redwater. The cause of this inability is really unknown, because such cattle infected, as they are, practically from birth by the tick, *Margaropus annulatus,* are highly resistant to piroplasmosis, but it is in all probability due to the scarcity of the piroplasm in the blood of such cattle, in spite of continual tick infestation. We do know that the blood of an animal which has recently suffered an acute attack of redwater, whether artificially produced or naturally acquired, is nearly always capable for some time after recovery of invoking a reaction in 100% of susceptible cattle, even though such animal be kept free of ticks *(Margaropus annulatus).* This is as we would expect, but with piroplasmosis, as it occurs in Queensland, this ability is frequently soon lost, though the period varies in individual cattle, and it is probably due to the disapperance to a large extent of the piroplasms from the blood, and with the disappearance of the piroplasms (as tested by inoculation of susceptible cattle) there is frequently a loss of immunity. Cattle which have suffered a natural attack of piroplasmosis, and then kept free of ticks *(Margaropus annulatus)* for as short a time as twelve months, and again reinfected, have been known to suffer a second natural attack of the same disease.

Under the circumstances, therefore, it was thought advisable to use as an immune animal for the engorgement of the ticks *(Haemaphysalis bispinosa)* one that had been recently immunized, and whilst engorging the ticks *(Haemaphysalis bispinosa)* on this animal, to test its blood during the same period as often as possible by inoculation of susceptible cattle. This was found to be a feasible proposition, because susceptible cattle are continually arriving from the southern States for inclusion in the herds of North Queensland, and these latter animals come to the experiment station for vaccination purposes.

Another matter that required consideration was the necessity of confining the ticks *(Haemaphysalis bispinosa)* somewhere on the body of the animals, and preventing their escape, or else placing the animals under such circumstances that, if the engorged ticks did fall to the ground, they would not be in a position to infect another suitable host. It was also necessary to collect several batches of engorged ticks, sometimes from the one animal after they had dropped off, so that they could be allowed to moult, or in the case of the adult, reproduce eggs and larvae, to be again used for a further infestation.

This method of collecting the ticks in batches, and allowing them to moult under artificial conditions, was considered as likely to be conducive of more satisfactory results than allowing the ticks to fall off naturally to the ground, moult there, and then bring about natural reinfestation of non-immune cattle which had access to the area. This last method would also depend on the possibility of the tick adapting itself at once to Queensland conditions, and would be on the whole an entirely unsatisfactory and haphazard method; and so, acting on the suggestion of the Director-General of the New Zealand Department of Agriculture, it was decided to use bulls in all the experiments, and to confine the ticks as far as possible to the skin over the testicles, which were covered with calico bags, so that engorged ticks on falling off would be caught and removed at leisure. This method proved to be entirely satisfactory, and the whole of the experiments have been conducted in this manner.

As the experiments were being conducted at Townsville, in the middle of country infested with *Margaropus annulatus*, it was essential that the non-immune cattle used should be placed under circumstances where infestation with this tick was impossible, and this was done by bringing the cattle by rail from non-infested country, and trucking them right into a paddock which had been cleansed of ticks *(Margaropus annulatus)* by systematic and continual dipping in the past. The bulls used in the various experiments were obtained on Afton Downs, country where the Queensland tick has never been known, and consequently cattle bred there are found to be highly susceptible to Redwater. These bulls have been kept for some months running in paddocks at the experiment station, and have never become infested with the Queensland cattle tick, and have remained in a condition of susceptibility to piroplasmosis.

Bull No. 1. This bull was selected for immunization, and passed through a typical redwater reaction in May, 1925. At first inoculation was carried out with blood from a locally bred animal, which had probably been infested with ticks all its life, and was certainly heavily infested at the time the blood was drawn, and on another occasion with blood from a second animal that had been kept free of ticks for some time, although originally infested. In neither case was a reaction produced, but the bull subsequently reacted to inoculation with blood from a third animal recently heavily tick-infested. Piroplasms appeared in the blood on the eighth, ninth, tenth, eleventh, and twelfth days after inoculation, and the ordinary clinical symptoms of piroplasmosis appeared, followed by recovery. This animal was then used during the subsequent six months for the engorgement of batches of *Haemaphysalis bispinosa*. During this time he was kept entirely free of *Margaropus annulatus*, but his blood was tested on the following dates in doses of 5 cc. in citrate solution.

Date.	Susceptible Animals Inoculated.	
15.7.25.	Cows 813, 814, 815, 815, 817	Typical reactions in all cases.
25.8.25.	Bulls 824, 825, 826	
7.10.25.	Bulls 838, 837, 839, 840, 841	

These were the only opportunities that occurred to test the blood of this bull on susceptible cattle during the period he was being used for the engorgement of batches of ticks *(Haemaphysalis bispinosa)*, but they are sufficient to indicate that his blood still contained the organism in sufficient numbers to invoke a reaction when inoculated subcutaneously.

Batches of larvae, nymphs, and adults *(Haemaphysalis bispinosa)*, all in the unengorged state, were required for engorgement on this animal. Some of these were obtained direct from New Zealand, but others had to be raised. To raise these "non-infected" batches of ticks *(Haemaphysalis bispinosa)* the non-immune cattle were used.

Experiment No. 1. To test the capacity of nymphs, engorged as larvae on an immune animal (Bull No. 1), to convey piroplasmosis when fed on a non-immune animal.

4.9.25. Five nymphs from larvae which had engorged on Bull No. 1 were placed on Bull No. 3. These nymphs commenced engorging, but were all dead by 9.9.25. This experiment was then discontinued, as no more nymphs were available at the time. Bull No. 3 was under observation for the subsequent three weeks, but showed no signs of illness.

16.11.25. Infected Bull No. 6 with 70 nymphs. From this experiment over 50 engorged nymphs were obtained, the last being removed on 23.11.25. Temperatures and blood examinations were commenced on 23.11.25, and continued until 11.12.25. A perusal of the temperature chart of this animal shows that a fairly high temperature was maintained, but it must be remembered that the season of the year was early summer, and the bull was driven in each morning to have the temperature taken. In semi-tropical countries like North Queensland, high temperatures such as those shown on the chart of this bull are not unusual. The blood examinations were entirely negative. The animal was kept under observation a week after cessation of the taking of the temperatures and blood smears, and showed no clinical signs of redwater.

Experiment No. 2. To test the capacity of adults to convey the disease, having engorged on a recovered animal in the previous larval stage, then on a non-immune animal in the subsequent nymphal stage.

Adults raised from the engorged nymphs obtained in Experiment No. 1 were used in Experiment No. 2.

8.12.25. Fed 50 adults on Bull No. 4.

14.12.25. Some of the ticks have apparently escaped from the bag, owing to its being torn the previous night, when many were almost replete and ready to drop off.

Only 7 fully engorged and 5 partly engorged were removed on 15.12.25.

Temperatures and blood examinations were commenced on 15.12.25, and continued until 3.1.26. The result was entirely negative. The animal was kept under observation for a week following, and was found to show no signs of clinical redwater.

Experiment No. 3. To test the capacity of larvae to convey the disease, after having fed on an immune animal in the previous larval stage, then fed as nymphs on a non-immune animal, and secondly as adults on a second non-immune animal.

Larvae raised from engorged adults obtained from Experiment No. 2 were used for this experiment.

16.1.26. Infected Bull No. 4 with several hundred larval ticks raised from adults obtained from Experiment No. 2. The bull was reinfected with more larvae on 21.1.26. Between 500 and 600 engorged larvae were obtained from this experiment, the last being removed on 25.1.26.

Temperatures and blood smears were obtained from this animal between 23.1.26 and 13.2.26, and showed no signs of a reaction.

"B" Series.

Experiment No. 4. To test the capacity of adults to convey piroplasmosis having fed as nymphs on an immune animal (Bull No. 1).

26.8.25. Fed 24 young adults on Bull No. 5.

1.9.25. Fed 15 young adults on Bull No. 5.

26.9.25. Fed 13 young adults on Bull No. 5.

From the above, 38 fully engorged adults were obtained, several others having been squashed by the animal lying down when the ticks were only partly engorged.

Temperatures and blood examinations were commenced on 2.9.25, and continued until 23.10.25. The result was entirely negative. Observation showed no clinical signs of redwater.

Experiment No. 5. To test the capacity of larvae to convey the disease, the parents having fed on a recovered animal in the previous nymphal stage, and then as adults on a non-immune animal.

Larvae raised from engorged adults obtained from the last experiment (No. 4) were used in this experiment.

13.10.25. Fed several hundred larval ticks on Bull No. 6.

15.10.25. All above larvae apparently dead by this date.

22.10.25. Fed several hundred larvae. Bull up to this date showed no signs of illness.

28.10.25. Fed a few hundred more fresh larvae.

6.11.25. From the above experiment about 900 engorged larvae were obtained altogether, and the attachment and engorgement of these ticks must be considered quite satisfactory. Last ticks were removed on 4.11.25.

Temperatures and blood smears commenced on 20.10.25, and continued until 19.11.25. The results were negative. The animal was kept under observation for a week following the cessation of temperatures and blood examinations, but no signs of redwater were evinced.

Experiment No. 6. To test the capacity of nymphs which had fed as larvae on a non-immune animal to convey the disease, the female parents having fed in their nymphal stage on a recovered animal, and then as adults on a non-immune animal.

Nymphs raised from engorged larvae from Experiment No. 5 were used in this experiment.

16.11.25. Infected Bull No. 5 with 100 nymphs.

From this infestation there were obtained 80 engorged nymphs, the last being removed on 23.11.25.

Temperatures and blood smears commenced on 23.11.25, and continued until 7.12.25. The result was negative. The animal was kept under observation for a further week. There was no sign of a reaction at any time.

"C" Series.

Experiment No. 7. To test the capacity of larvae to convey the disease, the parents having engorged in the adult stage on an immune animal.

26.9.25. Fed several hundred larval ticks on Bull No. 4.

30.9.25. All the above ticks apparently dead. None had engorged. Probably due to the hot weather.

1.10.25. Several hundred larval ticks placed on testicles.

5.10.25. Temperatures and blood examinations commenced. About 200 engorged larvae removed from bottom of bag.

13.10.25. Placed several hundred larvae on testicles.

15.10.25. Ticks placed on bull on 13th apparently dead. Infected with more larval ticks.

22.10.25. Many engorged ticks dead in bag. About 50 live engorged.

6.11.25. Again infected several hundred fresh larvae. From this last infestation about 400 engorged larvae were obtained, the last being removed on 11.11.25.

From this experiment about 850 engorged larvae were obtained.

Temperatures and blood examinations continued until 28.11.25. These were entirely negative, and further observation of this bull indicated no sign of a reaction.

Experiment No. 8. To test the capacity of nymphs to convey the disease, the parents having fed in the adult stage on a recovered animal, and the larvae on a non-immune animal.

Nymphs raised from the engorged larvae obtained from the last experiment (No. 7) were used in this experiment.

27.10.25. Fed 31 nymphs on Bull No. 3.

From the above experiment 23 engorged nymphs were obtained. Some nymphs had escaped from the bag. Last ticks were removed on 2.11.25.

Temperatures and blood smears taken from 2.11.25 to 22.11.25 were entirely negative.

The above experiment was then repeated as follows:

23.11.25. Fed 250 nymphs on Bull No. 3.

From the above 86 engorged nymphs were obtained, the last being removed on 28.11.25.

Temperatures and smears taken from 30.11.25 to 14.12.25 were negative. The bull was kept under observation for another week, but evinced no signs of a reaction.

Experiment No. 9. To test the capacity of adults which, in the larval stage had engorged on a non-immune animal, and as nymphs on a second non-immune animal, to convey the disease, the parents having engorged in the adult stage on an immune animal.

Adults raised from the engorged nymphs obtained from the last experiment (No. 9) were used in this experiment.

8.12.25. Fed 80 adults on Bull No. 5.

From this experiment 53 engorged adults were obtained, the last being removed on 17.12.25.

Temperatures and blood smears were commenced on 15.12.25, and continued until 3.1.26.

There was no sign of a reaction at any time. The animal was kept under observation for a further week.

DISCUSSION.

A perusal of these experiments, which were all negative, indicates at once that the most important are Nos. 1, 4, and 7, whilst possibly some importance might be ascribed to Nos. 2, 5, and 8, but it is scarcely likely that positive results could be expected from Nos. 3, 6, and 9.

It seemed, then, that all that was now required to be done was to test the susceptibility of the various non-immune bulls used for engorgement of the "infected" ticks.

However, this was not done at once, the reasons being as follows:

It has already been pointed out that the blood of immune Bull No. 1 was tested as often as possible for virulency during the six months over which it was used for "infection" of the ticks *(Haemaphysalis bispinosa)*, and was found on all occasions to invoke a reaction in susceptible cattle. These were the only cattle available at the time, and they were all bulls or cows imported for stud purposes from areas non-infested with *Margaropus annulatus.*

This seemed proof enough that the blood on which these ticks *(Haemaphysalis bispinosa)* engorged was infected with piroplasm, but it must be remembered that ticks only engorge a relatively small quantity of blood, especially larvae and nymphs, as compared with the amount of blood used for inoculation purposes (5 cc.). The possibility must therefore be borne in mind that a large number of ticks raised on this bull (No. 1) might not carry any, or enough, of the piroplasm to convey the disease, because, relatively speaking, we were only dealing with a small number of ticks in each experiment.

We do know, of course, that some tick-borne diseases can be conveyed experimentally by one tick, and theoretically this should be sufficient with piroplasmosis in Queensland. But with *Margaropus annulatus* in this State, experience suggests that a large number do not carry the piroplasm, because the tick has been known to appear in a previously non-infested area, and amongst non-immune cattle, and yet piroplasmosis has not followed, at least, not immediately. It has been assumed that the appearance of such "clean" ticks in previously infested areas has been brought about through the agency of horses, because *Margaropus annulatus* can, and does occasionally, infest and reach maturity on both horses and sheep, and it is believed that the larval tick on biting the horse "cleanses" itself, and that its progeny are clean, i.e., free from piroplasms. It is probably quite correct that the larvae raised from adults engorged on horses do not produce piroplasmosis when placed on susceptible cattle, but there is another reason altogether by which we can probably account for the introduction of ticks into previously non-infested areas *without accompanying piroplasmosis*.

It has been stated earlier in this paper that there is evidence to show that the blood of Queensland cattle bred on tick-infested country, and more or less heavily infested with ticks all their lives, will frequently fail to invoke a reaction when inoculated into susceptible cattle, and the suggestion is put forward that this is due to the small number of organisms in their blood. In fact, the small number of experiments carried out by ourselves shows that the majority of such animals are quite useless for bleeding purposes, as their blood is quite avirulent, even in fairly large doses. When it is remembered that the tick engorges a small amount of blood compared with that used with a hypodermic syringe, it is quite easy to understand why so many of their progeny will be non-infective. This, of course, is assuming that the piroplasm does not multiply, or if so, at least not to any considerable extent, in the body of the tick. We have no direct evidence on this point, because we do not know the life history of the piroplasm, neither have any experiments been conducted to test the capacity of small numbers of the larvae, the progeny of adults raised on such animals, to convey the disease in Queensland, because susceptible cattle are not easily available, at least, where we are situated. But we have other evidence to support our view. We know that butchers' cattle, brought in from clean country to the tick-infested country on the coast, frequently do not show evidence of piroplasmosis for some weeks after being placed in paddocks on the coast, even though they become tick-infested within a day or so of their arrival. This idea is further borne out by many of the older inhabitants, who remember the first invasion of the ticks, and who state that the tick frequently appeared some time before the accompanying redwater.

In a report written by Dr. Sidney Hunt, Government Pathologist, to the Chief Inspector of Stock for Queensland, concerning experiments which he (Dr. Hunt) had carried out on redwater, reference is made to certain Boolburra cattle used in the experiments. The report says: "These cattle (Boolburras), though grossly infested for some time, had never had Texas fever amongst them, although they were themselves (as was afterwards shown) as susceptible to that disease as clean cattle."

It cannot be regarded as presumptious to assume that many of the ticks which occur in the tick-infested areas of Queensland are not infected with *Piroplasma bigeminum*, simply because the organism is so rare in the blood of cattle immune to piroplasmosis, and it is such ticks, at first probably introduced in very small numbers, that are really responsible for the outbreaks of ticks, without accompanying piroplasmosis, in fresh areas. There is therefore no need to assume the spread of such ticks by means of horses. That cattle can easily wander across quarantine lines, which are not even properly fenced, or have no natural boundary, in some of the sparsely settled areas of Queensland, needs no further comment.

It might be possible, therefore, that although every precaution was taken to test the blood of the immune bull (No. 1), the reason for the failure of *Haemaphysalis bispinosa* to convey the disease was that the ticks were not picking up the infection, as it were, in sufficient quantities at least, to convey the disease to the non-immune animals. This possibility would not be so great with this particular animal (No. 1), because his blood was proved infective on all occasions used, whereas the blood of many animals bred and reared on tick-infested country in Queensland, and tick-infested all their lives, is frequently quite avirulent, even in large doses.

Still the amount of blood from Bull No. 1 used in each inoculation would be equal to that picked up by a fairly large number of ticks, and probably more than that picked up by the greatest number used in any experiment.

The best method of further testing the whole question was to repeat those experiments likely to prove positive, using immune animals, other than the particular one used in the experiments already concluded, for the engorgement of the ticks. A second bull (No. 2) was therefore immunized. It was inoculated with 10 cc. blood from Bull No. 837, and passed through a typical reaction. Piroplasms only appeared in the blood on one day, the twelfth, and could not be found at any other time, even after prolonged searching. On the fourteenth day, nymphs and adults were placed on the testicles of this bull, and with the engorged nymphs and adults obtained Experiments Nos. 4a and 7a were repeated. These were simply repetitions of Experiments Nos. 4 and 7.

Experiment No. 4a.

5.1.26. Fed 12 young adults on Bull No. 6.

9.1.26. Fed 20 young adults on Bull No. 6.

From the above experiment 11 fully engorged adults were removed, the others being partly engorged and squashed in the bag. Last ticks removed 16.1.26.

Temperatures and blood examinations between 12.1.26 and 30.1.26 showed no signs of a reaction.

Experiment No. 7a.

16.1.26. Infected Bull No. 5 with larval ticks.

Daily examinations showed that a number of ticks escaped from the bag at the neck. On 18.1.26 the calico bags were found off this bull. On 20.1.26, 23 engorged larvae were removed. On 21.1.26 this animal was reinfected with several hundred more larvae. On 25.1.26 50 more engorged larvae were removed. Although but few ticks were actually found in the bags, the engorgement of ticks on the animal was quite satisfactory. A rough estimate would be 500 to 600 engorged larvae. Many were removed in a partly engorged state, and many others must have fallen into the bedding.

Temperatures and blood examinations were taken between 23.1.26 and 15.2.26, and proved negative.

After these experiments had proved negative, an attempt was made to immunize a third bull (No. 3), but this animal died on the fourteenth day after inoculation from acute piroplasmosis, when about 20% of the red blood corpuscles were invaded with piroplasms, and before any ticks could be raised on this animal.

A further attempt was then made to immunize a fourth bull (No. 6). Piroplasms appeared in the blood on the ninth, tenth, eleventh, twelfth, and thirteenth days after inoculation, and on the twelfth day a batch of nymphs was placed on the testicles, and commenced to engorge, whilst piroplasms were still demonstrable in the blood by microscopic examination. These engorged nymphs, after moulting, were then used to repeat Experiment No. 4 (Experiment No. 4b).

Experiment No. 4b.

24.3.26. Infected Bull No. 5 with 12 young adults. Nine of these fully engorged, and three only partly engorged, the last being removed on 7.4.26.

Temperatures and blood smears were taken until 12.4.26, and the animal subsequently kept under observation. There was no sign of a reaction.

It was hoped to raise batches of larvae adults on this bull to repeat Experiments 1 and 7, but these all died before they reached maturity, although they commenced to engorge. The weather was exceedingly hot at the time, and the ticks seemed to object particularly to confinement within the bags during hot weather. Before other batches could be engorged this bull also died as the result of the inoculation. Incidentally, the reactions followed by death, which Bulls Nos. 3 and 6 gave to inoculations of blood from a recently recovered animal, showed their susceptibility to piroplasmosis at the time they were used for previous experiments.

Only two non-immune animals (Nos. 4 and 5) remained, and as there was little prospect of obtaining others, and as they had been used in some of the previous experiments as non-immune animals, it was necessary to test their susceptibility. Besides, the drought conditions prevailing at the time precluded any possibility of obtaining further animals for experimentation. These two bulls were therefore each inoculated with 10 cc. blood from a recently recovered animal, and both passed through typical reactions.

CONCLUSIONS.

1. The engorgement of *Haemaphysalis bispinosa* in each of the different stages of its life history on an animal recently recovered from piroplasmosis, followed by engorgement on a different susceptible animal in each of the three subsequent stages of its life history, failed to convey the disease.

2. All the susceptible cattle subsequently reacted to inoculation with virulent blood.

3. The experiments point against the possibility of *Haemaphysalis bispinosa* acting as the intermediary in the spread of bovine piroplasmosis *(Piroplasma bigeminum).*

THE MILK OF THE MONOTREME—*ECHIDNA ACULEATA MULTI-ACULEATA*

by

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The most primitive milk-producing animals existing are the monotremes, the only two genera of which are represented by the Echidnidae (native porcupines or spiny ant-eaters) and the Ornithorynchidae (the duck-billed platypus). They possess the essential structural characteristics of the mammalia. Their skeleton is distinctly mammalian, though with certain reptilian reminiscences, especially in the limb girdles, and the reptilian condition is still preserved in the fact that the genito-urinary system and the rectum open into a common cloaca.

The monotremes retain the most striking traces of their proximate ancestry in their mode of reproduction, as they lay eggs, which they hatch out either in a nest or an incubatorium pouch, instead of nourishing the foetus in utero by means of a placenta.

The Echidna transfers the egg to its ventral pouch, which in the breeding season becomes a functional incubatorium, where it very soon hatches, and the embryo obtains its nourishment by nuzzling the milk secreted from the many openings of the mammary ducts, which are scattered over the surface of the breast in the region of the pouch (1). As there is no development of a definite teat in the monotremes, it is practically impossible to collect milk from the living animal. The specimen from which the milk was obtained came from Rocky River, Kangaroo Island, and was of the species *Echidna aculeata multi-aculeata* (Rothschild, 1905).

The young Echidna was removed from the pouch on October 1, and the animal killed by chloroform fourteen days later. One-half hour after death, on incising the gland on one side, a considerable quantity of milk exuded, whereupon Professor F. Wood Jones suggested that a sample might be collected from the other side. The skin was incised and lifted, exposing the mammary gland. The capsule was then incised, and the contents squeezed towards the opening, where the milk was collected by suction, with little, if any, contamination with blood; 8·5 cc. in all were collected.

Methods of Analysis.

The *specific gravity* was determined in a 5 gm. S.G. bottle, and was 1·023.

The *proteins* were separated, and analyzed as follows:

1. *Casein.* One cubic centimetre (by means of an Ostwald pipette) was diluted to 5 cc. with distilled water, and ·02N citric acid added drop by drop until the casein flocculated; 2 cc. of phosphate-citrate buffer at P_H 4·7 were then added, and the sample made up to 10 cc. It was then centrifuged at high speed for 30 minutes, the supernatant liquid separated, and the nitrogen content of 1 cc. estimated by the micro method of Folin. The precipitate was soluble in dilute alkali, and precipitated from solution at a hydrogen-ion concentration approximating $10^{-4 \cdot 7}$, i.e., about the iso-electric point of the casein of cow's milk. Owing to the smallness of the sample available, it was impossible to further identify the nature of this protein fraction, but it is a reasonable assumption to class it as casein.

2. *Albumin and Globulin Fraction.* Five cubic centimetres of the supernatant liquid from the casein estimation was sealed in a hard glass tube, and immersed in a boiling water bath for two minutes. The tube was quickly cooled, and the contents transferred to a stoppered centrifuge tube, and spun at high speed for half an hour. The nitrogen content of this supernatant liquid was determined. Total nitrogen of a 1·10 dilution of the whole milk was similarly estimated. The nitrogen content of the different fractions was calculated by differences:

	Nitrogen per 100 cc. of Undiluted Milk.
Total	2·12 grms.
Total—Casein	0·92 grms.
Total—Casein—Albumin, Globulin (that is, non-protein N.)	0·34 grms.

3. *Carbohydrate.* One cc. milk was diluted to 10 cc., 2 cc. of ⅔ normal H_2SO_4 was added, and 2 cc. of 10% sodium tungstate, and the whole made up to 20 cc., and filtered. The filtrate was treated in a similar manner to a blood filtrate in the new Folin method for estimation of blood sugar. It was, however, read in the colorimeter against the colour produced by the reducing action of a standard lactose solution prepared from purified and desiccated lactose. The content of lactose was 2·81%.

4. *Identification of Carbohydrate.* The protein-free filtrate was added to the supernatant liquids from the protein analysis, boiled, filtered, and concentrated to about 2 cc. on a water bath; 2 drops (⅕ cc.) of phenylhydrazine base were added, together with 2 drops of glacial acetic acid, and the mixture placed in a boiling water bath for 30 minutes. It was then allowed to cool. The osazone was separated by centrifuging and recrystallized from dilute alcohol. The microscopic appearance of the osazone was definitely that of lactosazone.

5. *Fat.* The fat was determined by extraction of 2·6336 grms. milk absorbed in a fat-free extraction thimble with ether in a Soxhlet apparatus. The dried fat was determined gravimetrically. It consisted of a colourless oil, and occurred to the extent of 19·62%.

6. The *Saponification Value* of the fat was determined by the addition of 20 cc. of standard ·2M alcoholic KOH directly to the fat (·8168 gms.) in the Soxhlet flask, and refluxing for 30 minutes, back titrating with ·05N sulphuric acid. The saponification value proved to be ·192, practically the theoretical value for pure triolein.

7. *Soluble Volatile Fatty Acid* was estimated by completely precipitating the fatty acid from the soap formed in the previous test with excess of ·05M H_2SO_4, 200 cc. of water was added, and the mixture distilled through an adequate trap and condenser until 110 cc. of distillate had been collected. This was filtered from the insoluble fatty acid, and 100 cc. of the filtrate titrated with ·05M KOH. The titration value was less than one drop of alkali of this strength, showing the absence of soluble volatile fatty acid.

The fat was almost certainly pure triolein. The fatty acid was liquid at the temperature of the laboratory (20°C.), and melted at approximately 15°C.

Total solids and ash were determined gravimetrically with the rest of the available sample by drying at 100°C. in a silica dish, and later ashing in a furnace. The total solids by estimation were 36·75%, and ash ·78%.

TABLE A.

		Protein									
	Specific Gravity.	Casein.	Albumin. Globulin.	Non-Protein Nitrogen.	Lactose.	Fat	Sapon. Value of Fat.	Sol. Volatile Fatty Acid of Fat.	Ash.	Total Solids	Total Solids. accounted for.
idna	1023	8·4	2·9	0·34	2·81	19·62	192	Nil	0·78	36·75	34·85
		Total Protein.									
bit (3) ..	1047	12·0		—	2·00	13·5	—	—	2·50	30·5	—
n (3)	1029	0·80	1·21	—	6·37	3·74	—	—	0·30	12·48	—
w (3)	1031	2·88	0·51	—	4·94	3·68	220–230	25–30	0·72	12·73	—

DISCUSSION.

It will be noticed, on referring to Table A, that the milk contains essentially the same constituents as that of the higher mammals. The main protein is undoubtedly a casein. The carbohydrate is the disaccharide lactose. It is together with the differentiation of the mammary gland tissue that lactose makes its appearance in nature. Galactose, one of its constituent hexoses, occurs in combination in the brain of the higher vetebrates, and almost certainly also occurs in similar nerve tissues of the Reptilia. The fact that lactose is a non-threshhold substance, and is passed out without reabsorption in the kidney, whereas glucose (the normal sugar of the blood) is reabsorbed, makes it possible that lactose is the only sugar available to the organism which could be passed in concentrated form through the cells of the secreting acini.

The fat is peculiar, differing from that of the cow (the milk fat which has been most studied) in that it evidently consists of pure triolein, and contains no triglycerides of the lower fatty acids (tributyrin, etc.). The high content of fat is extraordinary when compared with that of the milk of man and of the cow, and it becomes evident from the ratio of carbohydrate to fat in the milk that the young Echidna must either store fat during the whole of the lactating period, or be endowed with the extraordinary power of α-oxidation of fatty acids. Otherwise extreme acidosis would follow from the accumulation of β-hydroxy butyric acid and other acetone bodies.

The baby rabbit is faced with the same predicament.

Assuming after Shaffer (2) that protein produces 20 millimols of antiketogenic substance and 10 millimols of diacetic acid per grm. of nitrogen, that fat produces $\frac{1}{3}$ mol of glycerol and 3 mols of diacetic acid per molecule of fat (the actual amount per grm. may be obtained from the saponification value of the fat in question), and that carbohydrate gives 5·56 millimols of antiketogenic substance per grm. of glucose, the approximate ratios of the milks shown in Table A are as follows:

Echidna	..	..	Anti : Keto :: 1 : 2·05
Rabbit	..	..	Anti : Keto :: 1 : 2·2
Man ..	..	..	Anti : Keto :: 2·1 : 1
Cow ..	..	..	Anti : Keto :: 2·5 : 1

In both the Echidna and the rabbit the complete oxidation of the milk-fat is impossible without the production of aceto-acetic acid in excess in the organism, and consequent acidosis.

SUMMARY.

The milk of Echidna does not differ in any extraordinary way from that of the higher mammalia; its constituents are the same, and their ratios fall within the limits of those found in the milks of other mammals. The absence of glycerides of soluble volatile fatty acids from the milk-fat is the only peculiarity which does not appear to have been noted in other milks.

I desire to thank Professor F. Wood Jones for his courtesy in collecting the milk and presenting the sample to the Department of Biochemistry.

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THE EFFECT OF A CONTINUED POLYURIA UPON THE KIDNEY OF THE RAT

by

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(Submitted for publication 13th November, 1926.)

In the course of a study of inositol excretion, Needham (1) produced extensive polyurias in rats, amounting at a maximum to 140 cc. of urine per rate per 24 hours. His animals survived 14 weeks, and at autopsy the kidneys were found to be completely disorganized. It was felt that the evidence for the latter contention was insufficient, and the symptomatology appeared to be unaccounted for by the findings, and in order to study the phenomenon, as well as to investigate the kidney function more closely, experiments were carried out as follows: Twelve adult rats were sustained on a normal balanced dietary of casein, potato, starch, butter, sucrose, and salt mixture, plus marmite, and to the mixture was added an excess of common salt, which was slowly increased until 15% was being ingested.

Hopkins' metabolism cages, holding six rats comfortably, were provided with a continuous water supply at two of the four chambers, and in the remaining two, packed tightly, and slightly moistened to assist binding, was placed the food. In this manner the animals could feed but not scrape the powdered food mixture into the cage, and so contaminate the urine. This apparently unpalatable diet was eaten with avidity, and only when the added salt reached 15% did one or two of the rats go off their food temporarily. Water was consumed continually, and a marked polyuria resulted. The faeces were separated from the urine by the usual glass bulb separator, and only in the extreme degrees of polyuria did they become soft and adhere to the collecting funnel or separator in any degree.

The appended chart shows the extent of the polyuria attained, and the point to be noted is the rapidity of readjustment following on resumption of a diet containing no excess salt, but identical in all other respects with the diet that preceded the change. There is some evidence that in the later stages the adjustment is not so rapid when the chemical constituents of the urine are examined, but the volume adjustment appears to be unimpaired so far as these experiments have gone. A long and detailed study of the chemical constituents and nitrogen partition has been made by one of us (M.L.M.), and will be reported later.

The animals not only maintained weight and condition, but also grew. There was no sign of oedema, either macro- or microscopically, nor by a study of weights after ceasing the salt diet, in fact, the rapid urinary adjustment corroborates this. The ratio of nitrogen/chlorine per cc. remained constant within the limits of error, indicating that fluctuations in dilution followed merely on fluctuations in food intake. There was no albumin in the urine at any stage. In order to prepare organs representing the condition of affairs at any stage in the experimental period, animals were killed and the tissues fixed in the following solution (2):

Potassium Bichromate, 40 grms.
Chromium Fluoride, 40 grms.
Water (distilled), 2,000 cc.

Sections were cut and stained by means of Mallory's phosphotungstic acid haematoxylin method, which has the advantage of showing up in a very marked degree certain granular appearances in the cytoplasm of the cells of the convoluted tubules (3). Others were stained by Scott's method (4), and do not show this feature, but show the absence of any interstitial change which might be expected after so long a period of strain. As may be seen from a study of the micro-photographs, there is no evidence that "the whole picture is one of enormous nuclear degeneration, together with general breakdown of cells," and all that one can say is that the cells are somewhat swollen and in a condition of great activity. The granules stained by the Mallory method show an interesting distribution almost entirely in the cells of the convoluted tubules, and, moreover, in these are packed densely towards the basement membrane of the cell. In the section from a kidney taken after the urine had returned to normal, these granules are seen to be distributed throughout the cytoplasm, and in most cases an arrangement of the granules in rows perpendicular to the basement membrane can be made out. These are undoubtedly the rows of fine granules forming the striations noted by Suzuki (5). According to Aschoff (6) the carmine injected in Suzuki's experiments did not stain these granules, but lay between the rows of granules prior to the final regression phenomenon of vacuole formation. These phenomena were interpreted as evidence against the reabsorptive activity of the cells, but recently Aschoff leans towards Mitamura's interpretation, the latter having shown that if in the rabbit a polyuria is induced in which secretion is increased 10 times, there is no deposit of carmine in the tubule cells, owing to the rapid passage of the urine down the tubules, the dye being present, however, by filtration through the glomerular capsule (7). In the present experiments, the mere concentration of the granules under the stress of the intense polyuria, in which secretion is increased 25 times, points to the passage of fluid towards the basement membrane. It will also be noted

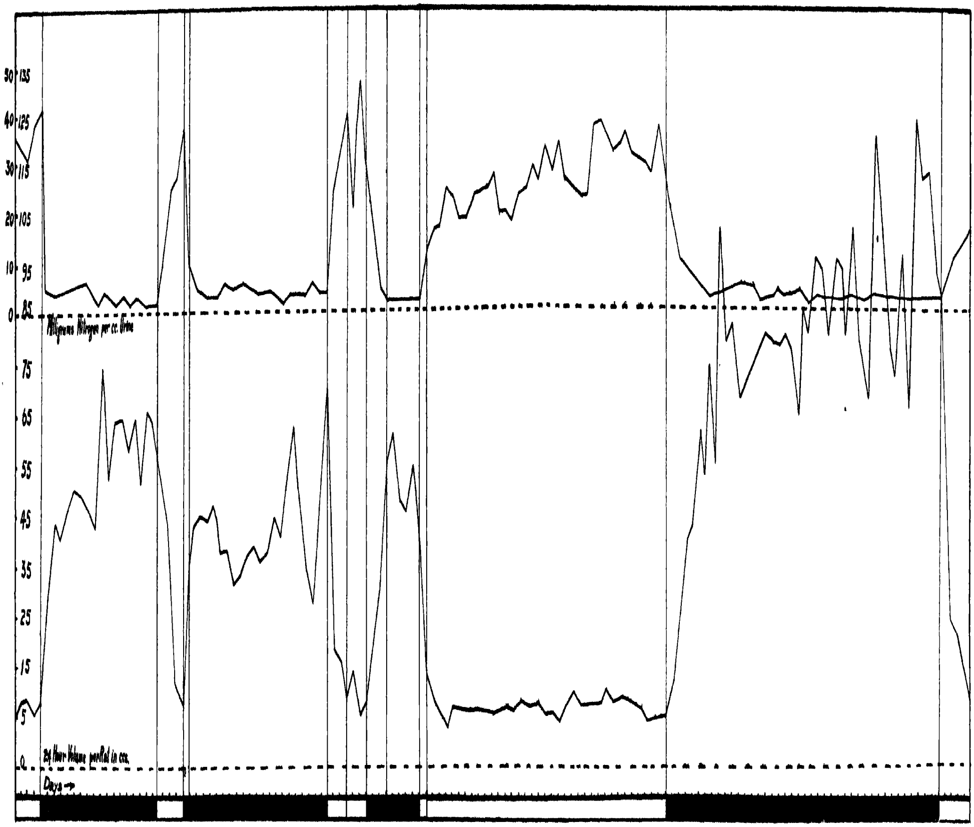

Chart showing secretion of urine by rats with artificially induced polyuria. The volumes are measured in cubic centimeters, and are subject to an experimentally determined position correction of approximately 5%, due to evaporation, from the collecting funnel, cage, etc. The whole period covered is from January 1, 1925, to June 2, 1925. The shaded sections represent periods during which the diet contained 15% excess of sodium-chloride, making a total ingestion of approximately 17% salts. Vertical lines are drawn at intervals for the purpose of analysis of the time relations between the curves.

that the tubes of Henle's loop are quite abnormally dilated, and this the more so by comparison with their fellow convoluted tubules. This may have a bearing on the possible pressure regulating mechanism suggested by various American and German authors (8), for it would appear that there is a collection of fluid in this situation between the primary and secondary convoluted tubules.

It is also evident that the glomeruli in extreme instances show evidence of some compression, and the interstitial spaces are wider than normal, the whole organ being very vascular. The liver shows evidence of this increased vascularity, as might be anticipated. It was friable, and crumbled when handled by the fingers in some cases, and in all cases weighed twice the normal weight. That this enormously increased flow through the portal area might have effects on liver function is to be expected, and there is evidence in favour of an increase in blood ammonia, which, owing to variations which seem to be related to the extent of hypertrophy of the liver, is at present under further investigation. The rest of the portal absorption area, apart from vascularity changes, shows little microscopic change, except the liver; where some increase of portal supporting tissue is evident, the epithelium and sub-epithelial tissue of the gut varying little, if any, from normal. When the rate of absorption and final excretion is considered in terms of the diameter of the tubes through which the fluid passes, the compensatory effort is the more to be appreciated. An interesting reflex of this is seen in the definite hypertrophy of the left heart.

SUMMARY AND CONCLUSIONS.

1. An experimental sustained polyuria of an extensive degree has been studied.

2. Over an experimental period of four months no evidence of impaired kidney function has been obtained.

3. Histological examination of the kidney shows no pathological change, all alteration being within the limits of normal.

4. The arrangement of cytoplasmic granules, staining by phosphotungstic acid haematoxylin, suggests active resorption from the lumen of the convoluted tubules towards the base of the living cells.

5. The livers of the experimental animals increased to double the normal size and weight, due to increased vascularity and possibly cell hypertrophy.

6. There is evidence of disturbance in urea formation, the blood ammonia being increased in the four animals so tested to six times the normal figure.

Acknowledgment is due to Dr. G. Scott Williamson, of the Dunn Laboratories, St. Bartholomew's Hospital, for assistance in preparing some of the sections; and to Dr. T. N. Seth, of Cambridge, for the analyses of blood for ammonia content.

This work was carried out during the tenure of one of us (C.S.H.) of a Beit Memorial Fellowship for Medical Research.

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Plate 1 shows section of cortex from rat passing 120 cc. urine per 24 hours after three months' poluria. Note the linear arrangement of the granules of the cytoplasm in the cells of the convoluted tubules and their deeper concentration towards the basement membrane. Compare the distension of the descending loop of Henle at X with that of the active associated convoluted tubules, where the lumen is more apparent than real, owing to the transparent nature of the cytoplasm as stained in this manner. The cell borders in the case of the "loop" epithelium are well defined, however, thus indicating less (if any) effect upon the latter than the former. Stained by phosphotungstic acid haematoxylin. Magnified ca. 300 diameters.

Plate 2. High-power view of section shown at Plate 1, giving in greater detail the indistinct nature of the cell border and its subjacent cytoplasm as stained by this method. The photograph is to be compared with Plate 8, in which there is some recovery from the effects of the polyuria. Stained by phosphotungstic acid haematoxylin. Magnified ca. 600 diameters.

Plate 3, showing cortex from polyuric rat during polyuria after four months' experiment. The method of staining shows the granules seen in the Mallory stained sections as white spaces. The cytoplasm generally takes up the stain, and the "brush" border cannot be distinguished as such. The distension of the descending limbs of Henle's loop can be compared with that of the convoluted tubules. The increased vascularity may be noted, as in the glomerular section. There is no increase of interstitial connective tissue. Stained by Scott's standard haematoxylin. Magnified ca. 600 diameters.

Plates 4 and **5.** High-power views from cortex of kidney from normal and polyuric rats respectively. Compare the distribution of granules in the cytoplasm of the convoluted tubules. Note that though the density of the photographs differs, the concentration of granules is greater towards the periphery of the polyuric tubular cells. Phosphotungstic acid haematoxylin. Magnified 400 diameters.

Plate 6. Section to show blood-vessel and interstitia. A glomerulus appears at X. The descending Henle loops are well seen to be dilated. There is no increase of interstitial tissue. Stained by Scott's standard haematoxylin. Magnified ca. 600 diameters.

Plate 7. Portal area from liver of rat after four months' polyuria, during active polyuria. Showing vascularity and some increase in portal interstitial tissue. The portal vein is dilated and engorged. The liver cells show no alteration either in cytoplasm or nuclei. Stained by phosphotungstic acid haematoxylin. Magnified ca. 150 diameters.

Plate 8. Section of cortex of kidney from experimental polyuric rat seven days after return to normal secretion after two months' experimentation. The dark staining granules are more widely distributed through the cytoplasm, and are not so concentrated towards the basement membrane. Scott's standard haematoxylin. Magnified ca. 600 diameters.

Plate 9. General view of normal rat kidney, showing selective action of stain upon the convoluted tubules. Compare with Plate 10 from polyuric rat kidney in fifth month of activity. Phosphotungstic acid haematoxylin. Magnified ca. 100 diameters.

Plate 10. General view of polyuric rat kidney in fifth month of activity. Note increased interstitial spaces and glomerular capsule space; other spaces not more dilated than normal. Staining and magnification as in Plate 9.

Plate 11. Showing a glomerulus and capsule from a rat at the height of polyuria after four months' experiment. The glomerular tuft will be noted to be very vascular, the half-tone discrete bodies being corpuscles. The intra-capsular space is not much greater than normal, and the capsule is not thickened. Scott's standard haematoxylin. Magnified 600 diameters.

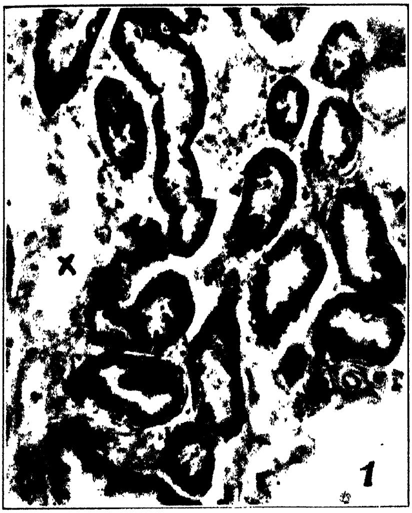

Plate 1.

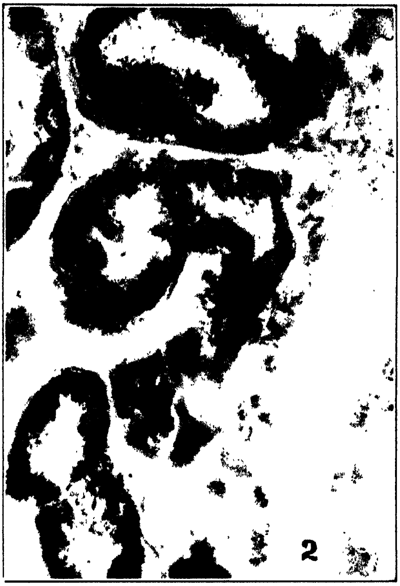

Plate 2.

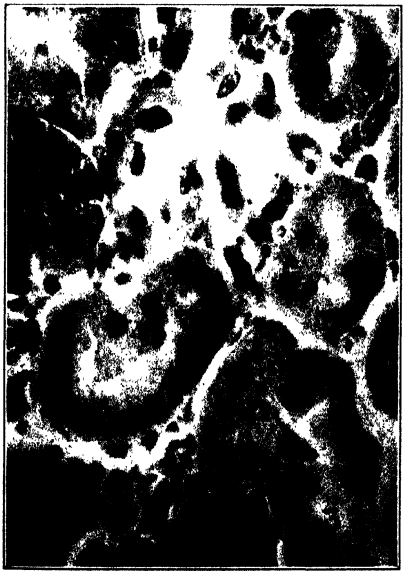

Plate 3.

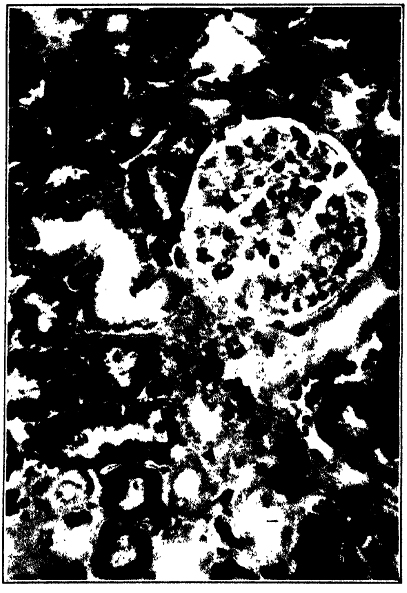

Plate 4.

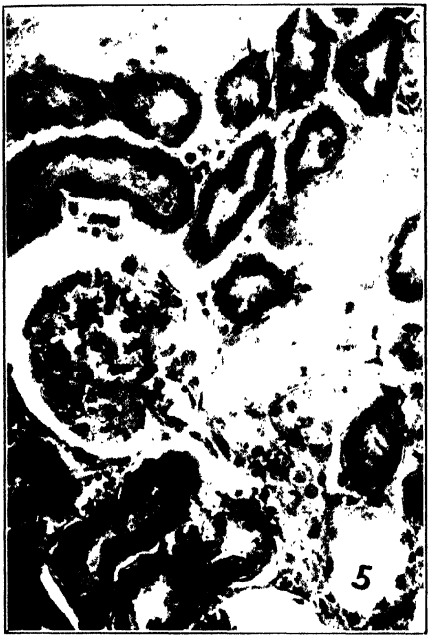

Plate 5.

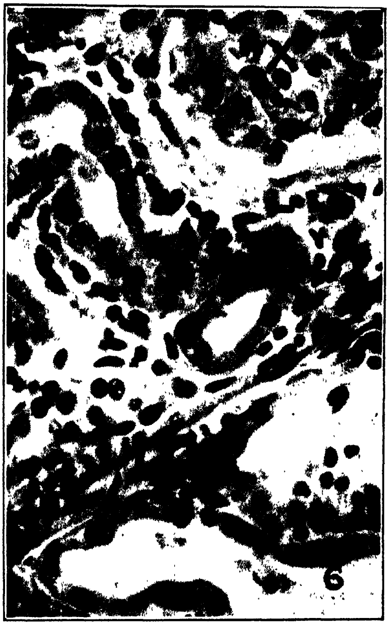

Plate 6.

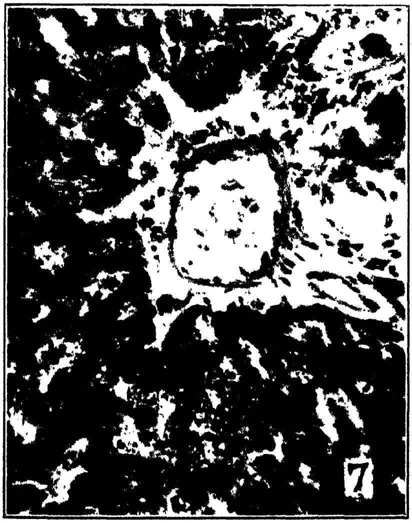

Plate 7.

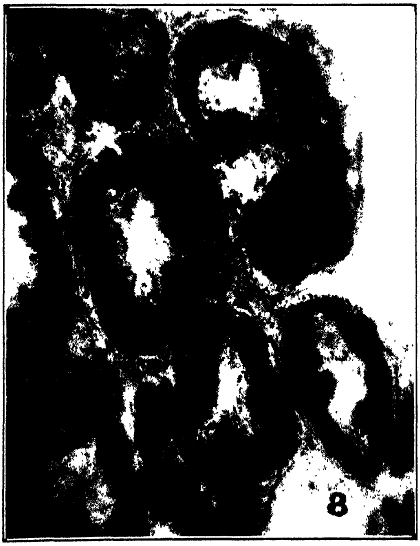

Plate 8.

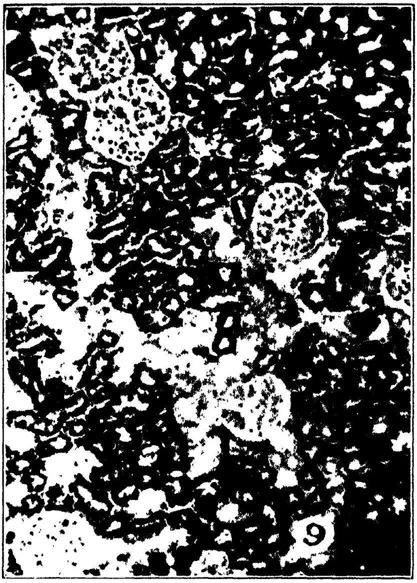

Plate 9.

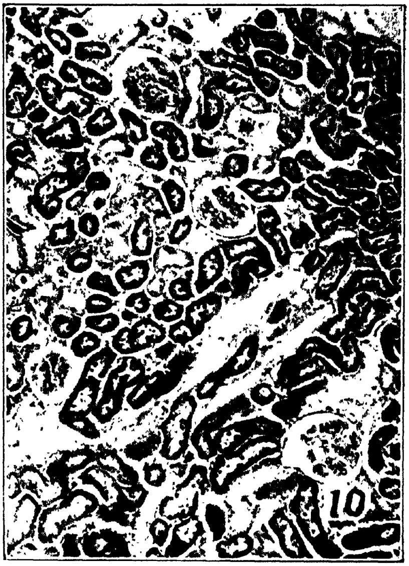

Plate 10.

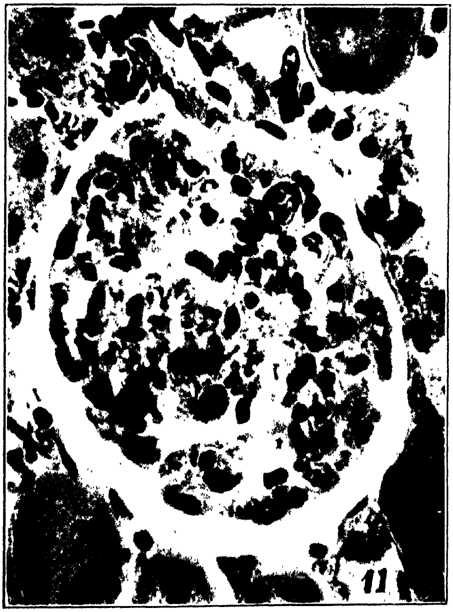

Plate 11.

PRELIMINARY NOTE ON THE TRANSMISSION OF THE EYE WORM OF AUSTRALIAN POULTRY

by

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(Submitted for publication 10th November, 1926.)

The occurrence of eye worms of poultry in Australia was first drawn attention to by H. Fern, Poultry Expert of the Queensland Government prior to 1907. Tryon (1907-8) drew attention to Fern's findings. Dodd (1909) recorded its occurrence in the Rockhampton district, and forwarded specimens to Sweet (1910), who described it as *Oxyspirura parvovum*. Tryon (1926) questions the validity of Sweet's new species, and states that it is not generally accepted. But until a comparison of a long series of this species and Cobbold's *Oxyspirura mansoni* has been made it is advisable to allow the Australian species to stand as at present. There is obviously a great variation in the size of worms, which is reflected in the measurements of the various organs, showing a corresponding increase or decrease as they depart from the average. Johnston (1910) recorded the occurrence of *O. mansoni* in New South Wales. Breinl (1913) and Nicoll (1914) both drew attention to the occurrence of *O. parvovum* in North Queensland, and the occurrence of eye worms in the muscovy duck, *Cairina moschata*, in North Queensland is here recorded. Dr. G. M. Heydon informs me of the occurrence of eye worms, which were identified as *Oxyspirura mansoni*, in fowls at Rabaul, in the mandated territory of New Guinea. According to Ransom, Dodd, and various other authors following them, this parasite occurs only on the sea coast; but this is not correct, at any rate so far as Queensland is concerned, as the present writer has found them in inland centres, viz., Charters Towers, Hughenden, etc. Ozoux (1910) recorded the occurrence of *O. mansoni* in the mountainous districts, as well as the coast of the Isle of Reunion. There have been various suggestions as to the mode of infection with eye worms. Emmerez and Megnin (1901) state that the eggs are laid outside the eye of the fowl, but do not state how, or give any evidence in support of their theory that development takes place in water. Ransom (1904) apparently performed a few experiments, with negative results. Ozoux (1910) appears to have carried out some work on the same lines as Ransom, but does not give details of his experiments. He states the worms die after 4–5 hours at 37°–38°, and disintegration takes place after 48 hours in the dark. He further states that the experiments tabulated allow him to consider that the eggs are hatched outside the body, probably on the soil, and are taken up by the host into the digestive tract, from which they reascend to the eye. He considers that the infection is not insect-borne. Emmerez (1918) states that the females lay their eggs in the eye, or are carried through the tear duct to the mouth, and thence into the intestine, where their bodies dissociate, thereby liberating the eggs, which are expelled in the stool, and hatch on the ground, the larvae living in the wet ground, eventually getting into the eyes of the birds when they take their dust bath.

In our observations, which have been carried out over a number of years, worms have been recovered alive in the mouth, oesophagus, and crop; and the eggs in the mouth, oesophagus, crop, along the alimentary tract, and in the droppings. These eggs hatched after varying periods from 24 hours upwards. The adults have been kept alive in various solutions for a number of days under laboratory conditions. Drinking water has been examined systematically after being centrifugalized, with negative results. The blood was examined on numerous occasions, but found to be negative in all cases for larvae. The eye fluid was also examined for the presence of eggs or larvae, with negative results. The pus-like material which is found in the eyes of infected birds was examined, and it was found that there are two kinds of matter: Firstly, a hard mass of old-standing material, and, secondly, a fresher and softer material, both of which, in our opinion, are the result of decomposition of the worms themselves. When a heavy infection takes place it is usually associated with catarrhal conditions, which block the passages; in consequence there is no outlet for the worms, which are in great numbers, and they eventually die and decompose. In the hard or old-standing material no eggs were seen in numerous examinations, but in the fresher and softer pus eggs were frequently recovered.

Examinations of the shady portions of the infected yard, where the fowls are in the habit of gathering together, revealed the fact that the ground was invariably riddled with filariform larvae, and showed a tremendous increase in the positive findings of nematode larvae over other portions of the yard. Concentrating on the possibility of the parasites being mechanically transmitted by flies, a number of bottle fly traps, containing various solutions, were set. These solutions were taken to the laboratory for examination. They were centrifugalized, and the sediment examined with a 1/6" objective. This was done daily for six weeks, but only a single nematode larva was seen, which was obviously not the worm under consideration.

An examination of a number of chickens and young ducks was carried out, and showed the presence of eye worms, in the case of the chickens after the age of 10 days had been attained, and in the case of ducks 14 to 21 days. This suggested placing a number of older birds, which had been given a course of treatment and kept under observation for three weeks and found negative,[1] in a special pen in an infected yard, with no precautions. After six weeks they were examined, and 83·3% were found to be heavily infected. In a somewhat similar experiment, but in this allowing the birds to mix with the other stock, and examining the eyes every three days, three birds were started; one was found to be infected after the expiration of 12 days, one after 15 days, and the remaining one became infected after 35 days.

The preceding observations tending to show, as they did, that the parasites were fairly well developed when introduced into the fowl, suggested a new line of attack, viz., that the infection was insect-borne, and an attack was made on the insect population of poultry yards.

The first to receive attention was the fowl tick, *Argas persicus*, but a long series of dissections and examinations proved negative; fowl lice, *Menopon* sp., gave the same results; the flies, *Sarcophaga* sp., *Musca domestica*, *Stomoxys calcitrans*, and a small unidentified fly also proved negative, as also did a large series of examinations of mites, *Dermanyssus gallinae*, and a number of crickets, *Nemobius* sp. Attention was then directed to the cockroaches, *Periplaneta australasiae* Fab. and *P. americana* Linn., and a number of adults and young forms gave negative results. But on concentrating on another species of roach, *Leucophaea surinamensis* Linn., we were immediately struck by the presence in the body cavity, in the nymphal and adult stages, of filariform worms, which appeared macroscopically and microscopically to be immature stages of the eye worm.

There were also usually associated with these worms numerous capsules containing larvae. These capsules varied in size as the development of the larvae proceeded in them, sometimes attaining 3 by 1·5 mm., of four measured accurately the measurements being 2·7 by 1·3 mm., 2·3 by 1·4 mm., 2·2 by 1·3 mm., and 1·8 by 1·1 mm.

On examining a long series of cockroaches, dividing them into three batches, the percentage of infection was as follows:

(*a*) Young and very young forms, no infection present.
(*b*) Intermediate forms showed an infection rate of 38%.
(*c*) Adult forms, an infection rate of 93%.

This gave a percentage infection rate of the total examined of 56%. The number of worms obtained from each cockroach averaged 21. In numerous individuals the total number recovered was small, viz., 2 and 3, but in one case a total of 108 worms and capsules were obtained, and in others similar high numbers were observed.

---

[1] This treatment was given irrespective of whether the birds were infected or uninfected before proceeding with an experiment, on account of the fact that almost all yards were more or less infected.

An examination of a number of cockroaches was carried out, with a view to obtaining information regarding the length of time the worms could live after the death of the insect. Some of the roaches were just on the point of dying; others had been dead for varying periods up to a maximum time of 64 hours. The examination showed that the activity of the worms was just as great in the roaches which had been dead the maximum time, even though in these cases decomposition was in an advanced stage. Further work is proceeding on these lines. Arrangements were then made to carry out experiments to prove whether the worms so obtained were eye worms. Young birds were obtained from an uninfected yard, and were kept under observation for some days prior to experiment. The young ducks were hatched in the ordinary way, but the young chicks were incubator bred.

Experiment 1. One duckling, about a week old, was fed with four cockroaches in the nymphal stage; it was examined 17 hours later, and found to be infected with eye worms.

Experiment 2. Four ducklings from the same batch as (*a*) were fed with three cockroaches, each in the nymphal stage, by pushing them whole down the throat, and when examined 17 hours later were all found to be infected.

Experiment 3. One duckling was given seven worms, extracted from infected roaches, the worms being placed on the tongue with a camel hair brush. On examination 17 hours later it was found to have worms in both eyes. Control ducks were kept, and remained negative during the time the birds were under observation.

Experiment 4. One duck, 17 days old, was given cockroach entrails with capsules attached (one worm was just emerging), the whole being placed in the mouth at 12.46 p.m.; when examined at 12.58 p.m., 12 minutes after introduction, worms were found in both eyes. Control negative.

Experiment 5. One duck, 17 days old, was given 5 worms, taken from cockroaches; 13 minutes after introduction into the mouth they were found in the eyes. Control negative.

Experiment 6. One duck, 17 days old, was given 10 cockroaches which had been etherized. It was examined 10 and 20 minutes afterwards. There being no sign of eye worms, a further 5 roaches were introduced, and the examination carried out at five-minute intervals. At the expiration of 55 minutes worms were found in both eyes. No control kept.

Experiment 7. Eight young chickens, 9 days old, were each given 4 whole cockroaches in the nymphal stage; a few extra roaches were then scattered in the box, and were picked up by the chickens. Two and a half to three hours afterwards the chicks were all infected. Controls negative.

Experiment 8. Three young chicks, 10 days old, were given 1 adult cockroach each, between 3.35 and 3.40 p.m. They were then constantly examined for the presence of worms in the eyes; this occurred at 3.58, i.e., 18 to 23 minutes

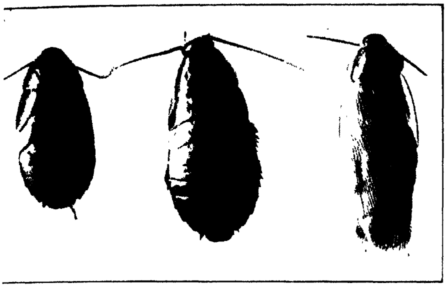

Fig. 1. × 1·7.

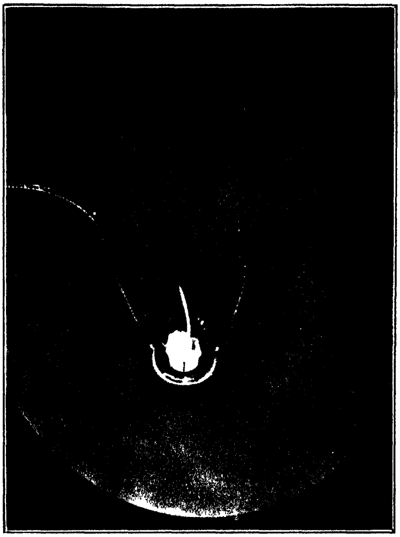

Fig. 3. × 3·8.

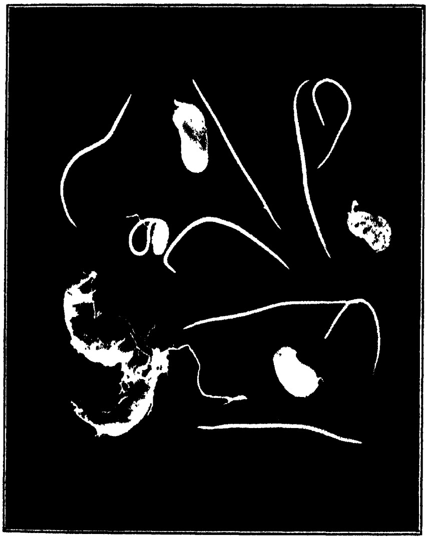

Fig. 2. × 3·8.

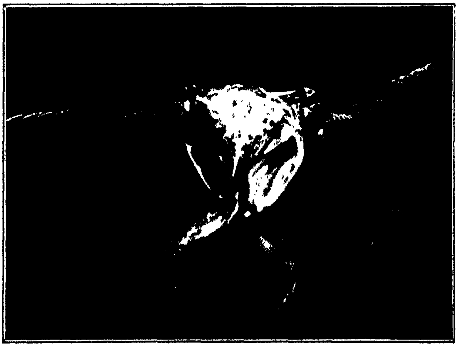

Fig. 4. × 3·8.

F. H. Taylor, Photo.

Fig. 1. Showing adult male and miniature females of cockroach, *Leucophaea surinamensis*, Linn.

Fig. 2. Showing worms, cysts, and portion of gut with cysts attached, taken from *Leucophaea surinamensis*.

Fig. 3. Showing head of *Leucophaea surinamensis* with worm protruding.

Fig. 4. Showing third pair of legs and portion of body of *Leucophaea surinamensis*, with two worms protruding.

after the introduction of the cockroaches. Care was exercised in introducing the insects to the birds, so as to ensure that the former should not be ruptured during the process. Control negative.

Experiment 9. One chick, 11 days old, was given 3 adult cockroaches, care being taken not to rupture them. The chicks were then kept under constant observation, and in exactly 17 minutes after the feeding worms were noted in the eyes. No control was kept.

Experiment 10. One adult muscovy duck, which was examined thoroughly for the presence of eyeworms, and found to be negative, was then given 8 cockroaches, taking care not to rupture them. The mouth and eyes were then kept under constant observation, and exactly 8 minutes after the introduction worms were seen on the roof of the mouth, and on the tongue, 9 worms in these positions being counted. After this the eyes were watched constantly, but worms did not appear until 31 minutes from the start, i.e., 23 minutes after their presence in the mouth. It was interesting to note that worms were seen on five occasions passing into the eyes from the naso-lachrymal duct. No control was kept.

It was thought advisable, in view of the theory that the cockroaches do not go beyond the crop before the worms are liberated, and considering the short space of time occupied between the introduction of the cockroaches and the appearance of the worms in the eyes of the bird, to note the fate and position of the cockroaches in an allotted time. For this purpose four young chicks were killed at varying intervals, viz., 5, 10, 15, and 30 minutes, after the introduction of the insects.

Experiment 11. A chick, 16 days old, was given 1 adult cockroach, and after an interval of 5 minutes was killed, the insect being found at the entrance of the crop, on the top of the contained foodstuff. On examination of the roach there was no apparent change noted. No free worms were observed.

Experiment 12. A chick, 16 days old, was given 1 adult cockroach, and after an interval of 10 minutes was killed. Except that the cockroach was in the middle of the foodstuff, no difference was observed. No free worms were seen.

Experiment 13. A chick, 16 days old, was given 1 adult cockroach, and after 15 minutes was killed, the roach being found at the entrance to the crop, on top of the foodstuff. The skin of the insect was observed to be in a fairly soft condition, and a worm was seen just emerging from the soft part of the under-surface of the body, between the third and fourth leg. No free worms were seen before dissection.

Experiment 14. A chick, 16 days old, was given 2 adult cockroaches, and after 30 minutes the chick was killed; the roaches were on top of the foodstuff, at the entrance of the crop, and were found to be very soft, and easily broken at the slightest touch with the dissecting needles. No worms were noted until the cockroaches were dissected, when a fair number were found.

It would appear, although only one escaping or escaped worm was seen in the preceding series of experiments, that the cockroaches do not pass beyond the crop within the limits of the time necessary for an infection to take place, and that the digestive juices are responsible for the softening of the tissues, and probably the increased temperature has a stimulating effect on the worms, which break through the softer portions of the body. This probably applies only in cases where the roaches were swallowed intact, which would occur only in a small percentage of cases, as it is very noticeable that the birds peck at the insect, and by so doing rupture most of them, thus making an easy exit for the escape of the worms.

From the foregoing experiments there appears to be indisputable evidence that the cockroach, *Leucophaea surinamensis* Linn., is a responsible agent for the transmission of the eye worms of fowls and ducks. The cockroaches apparently ingest the young larvae from the droppings and from the ground. Having attained their objective, the larvae then pass through the wall of the alimentary tract, on the outside of which they encapsulate. In the capsules development takes place, and on attaining the stage of infectivity, they leave the capsules, and wander about in the body cavities. They have been recovered from both the thoracic and abdominal cavities, as well as the legs. The infected cockroaches are in their turn taken up by the poultry, and in our opinion do not pass further than the crop before the worms which have attained the infective stage escape from the intermediate host; the parasites then pass up the oesophagus to the mouth, and eventually through the naso-lachrymal duct to their destination. There now remains to examine in detail two stages in the life history of this parasite. It is anticipated that this will take come considerable time to complete, as in (*a*) it will be necessary to feed one or two fowls with large numbers of infected cockroaches, so that heavily infected birds may be available for ascertaining the life history of the worm in the fowl, particularly the number of ecdyses and changes that occur in the worms before they attain maturity and reproduction begins, and (*b*) to obtain information regarding the time taken by the larvae to reach the infective stage after entering the cockroach.

It would appear that there are five distinct stages in the development of this parasite, quite apart from any changes which occur during each stage:

1. Embryonic stage, portion of which is spent in the parent worm, portion in the alimentary tract of the bird, and portion on the ground.
2. Larval stage, portion of which is spent in the ground, and portion in the alimentary tract of the cockroach.
3. Cystic stage, in capsules on the outside of the alimentary tract.
4. Free or infective stage, in body cavities, legs, etc.
5. Pre-adult and adult stage, in the eyes of the birds, where development is completed.

Treatment.

The first necessity in getting rid of eye worms, now that there is a known intermediate host, is obviously a concentrated attack on all cockroaches, and it would appear that the best method is trapping, since poisoning and fumigation are too dangerous to poultry. The yard should be cleaned of all droppings, and sprayed well with a good disinfectant frequently. The poultry should then be treated, preferably by placing a few drops of turpentine in the eyes, and allowing it to remain for half an hour, then irrigating with lukewarm water or boracic water, and removing worms from the eyes with a small camel hair brush. Although this treatment is undoubtedly severe, it has the advantage of being quick in action. Weak Condy's fluid is also recommended, but is much slower, and requires some days to obtain the required results. Some poultry-keepers use kerosene, and find that it gives good results.

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I wish to acknowledge the assistance of Mr. G. Brindle in supplying material; Mr. and Mrs. M. Gilmour for allowing free access to their poultry yard, and whose kindness is largely responsible for the results obtained.

My thanks are also due to Mr. F. H. Taylor, of this Institute, for the photographs and identification of the male cockroaches; and to Mr. Henry Hacker, of the Queensland Museum, for identifying the females.

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ON THE ACCELERATION OF ENZYMATIC SYNTHESIS OF PROTEINS BY LIPOIDAL EMULSIONS

by

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(From the Darling Laboratories of Physiology and Biochemistry,
University of Adelaide).

(Submitted for publication 13th November, 1926.)

The fact that the proteolytic enzymes are able to catalytically resynthesize protein from its hydrolytic products was pointed out concurrently by T. Brailsford Robertson (3, 4) and A. E. Taylor (6). To demonstrate the reversal of the hydrolytic activity of the enzyme Robertson utilized pepsin and the peptic digestion products of casein, and Taylor, a tryptase from the liver of the Californian clam, *Schizothoerus nutalli*, and the tryptic digestion products of the simple protamine salmin. Robertson, in collaboration with Gay (1), was able to prove that the product of the reaction was antigenic, and identical with casein or one of its early cleavage-products. He also showed that the synthetic reaction velocity was increased by heating above the optimal temperature for the hydrolytic activity of the enzyme, and that a high concentration of enzyme favoured synthesis, the former fact leading him to consider that the synthesizing enzyme was a tautomeric anhydride of the hydrolyzing enzyme.

Wasteneys and Borsook (7) have recently confirmed the earlier findings of Robertson, and extended our knowledge by demonstrating the importance of the hydrogen ion concentration in the reversal. They showed without doubt that the synthesized material was of the same order of complexity as the native protein.

The remarkable rapidity with which the living cell is able to repair itself after inanition, and to synthesize specific products during proliferation, clearly points to the occurrence within the cell of a physico-chemical mechanism which favours synthetic activity. This has lead to the hypothesis that such a site exists in the mitochondria (2), the water poor phases existing at the surface of the colloidal lipoids in these bodies instituting a favourable set of conditions for the enzyme to exert its synthetic activity.

More recently Robertson (5), in extending this hypothesis, pointed out that the lipoids would tend to orient the molecules of amino acids at the surfaces, and bring the reactive amino and carboxyl groups into close proximity, and to point them towards the aqueous phase, whereas the lyotrope hydrocarbon chains would be buried in the lipoid phase. The effect of thus concentrating and bringing into close proximity the reactive groups would be to favour synthesis, even if the catalyst were not in high concentration at the synthetic site, but as we are led to believe from other evidence (2) that the proteolytic enzyme is concentrated in the mitochondria, wherein the main lipoid phases of the cytoplasm of the cell occur, it becomes clear that practically ideal conditions for the building up of proteins exist in these bodies.

Before the latter publication (5) appeared the author had observed in several experiments that the rate and completeness of resynthesis of protein complexes from the hydrolytic products of casein by the action of pepsin were greatly influenced if emulsions of lecithin and cholesterol were added to the reaction mixture, the lipoid emulsions tending to make the final production of protein-like bodies more complete, and to increase the reaction velocity of the synthesis.

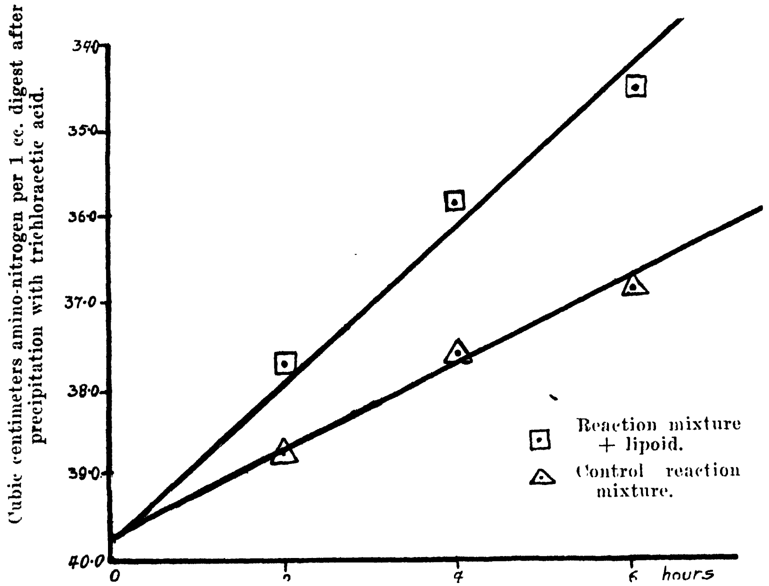
That lipoid emulsions also increase the reaction velocity of the synthetic activity of trypsin is evident from the following experiment:

A substrate was prepared by digesting a 5% solution of egg albumin in a ·1 mol. phosphate-citrate buffer with trypsin, separated from pigs' pancreas, for several days, until a complete absence of protein was indicated by the failure to produce a precipitate with trichloracetic acid. The digest was boiled for five minutes to inactivate the enzyme, and was then concentrated to one-fifth its bulk in a serum drier at 40°C. The solution was preservatized throughout by the addition of toluol. It was adjusted to P_H 5·8, which Wasteneys showed was approximately the optimum reaction for the tryptic synthesis, by first establishing its titration curve against ·1 normal citric acid by means of the gold-quinhydrone electrode, the potentials being estimated with a high precision potentiometer equipment.

The trypsin employed was separated from acetone-exhausted pig's pancreas by extraction with water, and after filtering the concentrated extract it was adjusted to P_H 7·5, and digested at 35°C. for 8 hours, and then dialyzed for 8 hours against a stream of distilled water in a close celloidin sack, into which was introduced a fairly high-speed mechanical stirrer. The enzyme was then either precipitated by the addition of 10 volumes of absolute alcohol or frozen in aqueous solution at —5°C., and stored until wanted at this temperature.

The lipoid emulsion was prepared from egg yolk by the following method: The yolks of fresh eggs were separated from the whites as completely as possible, and stirred into twice their bulk of anhydrous sodium sulphate. After drying, the mixture was powdered and extracted in a continuous extraction apparatus with ether. The ether was then removed from the extracted fats, and the residue was taken up in alcohol, so as to obtain a solution of about 30% fat. This solution was poured into water whilst stirring with a high-speed electric stirrer. By this procedure a very stable emulsion may be prepared, containing upwards of 20%

fat in a condition which is remarkably stable to changes in hydrogen ion concentration, and change in concentration of neutral salts. The alcohol is separated by distilling off under reduced pressure at 40°C. Pure lecithin emulsions were discarded in favour of those of mixed egg lipoids, owing to the extreme lability of the former.



Two 50 cc. portions of the substrate were adjusted to a reaction of P_H 5·8 by the addition of the required amount of citric acid, as calculated from the titration curve of 10 cc. of the same substrate previously determined. These were then introduced into long-necked, stoppered flasks, which were immersed under the surface of the water in a thermostat at 40°C. (± ·01°C.). To one, 5 cc. of water was added; to the other 5 cc. of the concentrated lipoid emulsion, and after the contents of the flasks had reached that of the bath, 1 cc. of 20% trypsin, which had previously been brought to the temperature of the bath, was added to each flask.

At the times indicated on the protocol 1 cc. was withdrawn from each mixture, and run into 10 cc. of 2% trichloracetic acid, and the whole made up to 20 cc., filtered, and the amino-acid content estimated at once in 2 cc. of the filtrate by means of a micro Van Slyke apparatus. The analyses were done in duplicate, and where the second differed more than 1% from the first estimate a third analysis was performed.

The results show clearly the increase in velocity of the synthetic action due to the colloidal lipoid phase. The synthetic reaction is practically linear over the range studied, but this represents but a small portion of the complete reaction. From the linear slopes the reaction velocity is almost doubled under the conditions of this experiment. Precipitated protein was noted in the reaction mixture containing lipoid after about 90 minutes, and appeared in about 150 minutes in the control digest.

SUMMARY.

Colloidal emulsions of egg fat greatly increase the reaction velocity of the resynthesis of protein from its digestion products by trypsin.

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ON SOME CONDITIONS AFFECTING THE VIABILITY OF CULTURES OF INFUSORIA AND THE OCCURRENCE OF ALLELOCATALYSIS THEREIN

by

T. BRAILSFORD ROBERTSON

(From the Darling Laboratories of Physiology and Biochemistry
of the University of Adelaide).

(Submitted for publication 11th December, 1926.)

1. *Conditions affecting the occurrence of Allelocatalysis.*

In the course of investigations carried out during the past several years upon the conditions affecting the reproduction of Infusoria in hay infusion, I found that the rate of reproduction is affected in two different ways by the density of the infusorial populations. In young cultures it was found that reproductive rate increased with density of population, while in old, thickly inhabited cultures the density of population appeared on the contrary to be an inhibiting factor, for reproduction ceases, although the culture fluid, after removal of the infusoria, is still able to support the multiplication of infusoria which are freshly inoculated into it (14).

The accelerative effect of increasing population in young cultures is evidenced in diverse ways. Thus the rate of production of new individuals in subcultures rises in an autocatalytic curve as the population increases. It was further found, moreover, that single infusoria, isolated into small volumes of fresh culture medium, reproduce more rapidly than infusoria from the same parent culture which are isolated into larger volumes of culture medium (15). This observation Cutler and Crump have been unable to confirm (4). Again, it was found that two individuals isolated together into the same small volume of culture medium reproduce more rapidly than a single individual isolated into a like volume of the same medium (allelocatalytic effect). This observation also Cutler and Crump have failed to confirm, while Peskett (10) has been unable to obtain any evidence of the occurrence of the phenomenon in cultures of yeast. Finally, I found that allelocatalytic effect is augmented and the reproductive rate diminished by washing the infusoria prior to their introduction into the culture (17, 18); these observations, furthermore, Cutler and Crump (6) and Peskett (10) have not been able to repeat.

Before entering upon a discussion of some possible origins of these discrepancies it is necessary to draw attention to the fact that the phenomenon of allelocatalysis is by no means confined to cultures of infusoria, but, on the contrary, appears to be of very general occurrence. Thus it has been shown by Burnet (1) to occur in cultures of bacteria. Fischer (7) has found that in connective-tissue cultures *in vitro* single fibroblasts do not multiply, growth taking place only when the cells are numerous and close to one another. Burrows and Suzuki have noted analogous phenomena (2). The occurrence of more than one autocatalytic cycle of growth in vertebrate animals shows that certain cells in the animal share a common catalyzor, while others, which are not influenced by this catalyzor, produce a growth-catalyzor of their own, which they share among themselves, with the result that their growth gives rise to a separate autocatalytic cycle of weight-increment (19). The effect of *failure* to share a common catalyzor in thus rendering the processes of growth independent of one another, shows that, when growth involving a multiplicity of cells and tissues yields weight-increment which is defined by a single curve of autocatalysis, all of the cells concerned must be affected by the growth of each of them, or in other words, that some common catalyzor is diffused throughout the whole mass, being produced by every cell in the group or community, and affecting the growth of each of them in a like manner. The growth of a bird or mammal consists of two or three mutually independent autocatalytic cycles, because these animals contain two or three such communities of cells, which differ from one another in the nature of the catalyzor which they share. Allelocatalysis, therefore, must occur in the growth of those communities of cells which comprise the higher animals and plants, since the growth of all of these which have been investigated seems to consist of one or more autocatalytic processes.

If, therefore, communities of yeast or infusoria should chance not to display allelocatalysis, their behaviour would constitute an exception to the usual behaviour of cell communities, and it would become of some importance to ascertain the origins of this departure from the general rule.

Before proceeding to describe the investigations which form the second part of this paper, it is necessary to dwell upon certain differences between the technique employed by Cutler and Crump and that which I have employed, since the disagreement of our results may originate in any of these, or in several of them combined.

In the first place, attention must once more be drawn to the fact that until the experiments were performed which are described in their most recent paper (6), Cutler and Crump did not employ washed individuals in their experiments upon allelocatalysis, whereas I have always washed the isolated individuals at least once (unless the contrary is specifically stated), and usually twice, in relatively large volumes of fresh culture medium before employing them to test for the mutually accelerative effect of pairs of individuals upon each other's reproduction. Cutler and Crump have endeavoured to insinuate that this was not the case, by quoting my words describing an experiment in which it is not specifically stated that the individuals employed were washed. Because it was not stated to have been done in this individual experiment it is not to be inferred that the washing was omitted, and, in fact, this precaution appeared to me, from the very first experiment of this nature which I performed, to be so very obviously a necessary one that it never occurred to me to omit it, except with the deliberate intent of ascertaining the effect of *failure* to wash the animals before subculturing them. Presumably, if contiguous animals can affect each other's growth without the occurrence of conjugation, they must do so through the agency of some soluble substance which they emit, and which is transferred from one organism to the other through the medium which they inhabit. Presumably, therefore, also, this soluble substance must be very abundant in the thickly inhabited cultures from which subcultures are usually prepared. Therefore the removal of adherent parent-culture medium from the bodies of the individuals employed for inoculation was an obviously necessary preliminary to the discovery of allelocatalytic effect, if any such effect occurred. Later investigations showed, in fact, that washing of the infusoria leads not only to removal of adherent parent-culture fluid, but also to washing out of accumulations of autocatalyst from the protoplasm of the cells themselves (17).[1]

Another difference between the techniques employed by Cutler and Crump and myself resides in the fact that instead of employing the "natural" medium, to wit, hay infusion, in which my observations have been made, they employ a "synthetic medium," originally devised by Peters to show that reproduction of protozoa could take place in an artificial medium and in the absence of associated bacteria (12). Instead of utilizing this discovery of Peters, however, and cultivating their infusoria in bacteria-free media, they introduce bacteria (Sarcina) into the medium as well, thus introducing the very complication (that of simultaneous growth of two organisms in the same medium) which the medium appeared to have been designed to avoid.

The results obtained by Peters, in fact, have failed to receive confirmation, and the observations of Cutler and Crump themselves indicate that the complication arising out of the growth of associated organisms is an unavoidable one, because non-parasitic infusoria will not grow in their absence. Thus Cutler and Crump have shown that the culture of *Colpidium* employed by Peters was contaminated by an unidentified species of small bacillus; that when these are scanty the ciliates show "typical signs of hunger degeneration," and that when they are abundant the growth of the *Colpidia* is greatly stimulated (5). Furthermore, it has been shown by Cutler and Crump that the multiplicative rate of *Colpidia* increases with the ratio of these bacteria to the ciliates inhabiting the medium.

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[1] In my first article on this subject it was shown that washing with distilled water prevents allelocatalytic effect. This, however, is entirely distinct from the effect of washing the infusoria with the medium into which they are subsequently subcultured.

Two possibilities now suggest themselves: If, on the one hand, the ciliates actually feed only upon these "contaminating" bacteria, then their growth must obviously determine the growth of the infusoria and the density of the original bacterial inoculum will determine the initial rate of growth of a subculture, not the number of infusoria. Thus two infusoria inoculated together into a given volume of culture medium may be prevented from multiplying more rapidly than a single individual, simply because the food-supply is inadequate to permit reproduction beyond a certain maximum rate, no matter what stimulating substances or catalyzors may be present. In all cultures, then, containing one, two, three, or more infusoria in the original inoculation, we may merely be obtaining a growth rate which is rendered uniform by the uniform magnitude of the original bacterial inoculation, or else a growth-rate in which the diminished bacteria-ciliate ratio counterbalances or overshadows the allelocatalytic effect due to the presence of more than one ciliate in the subculture.

If, on the other hand, the infusoria can live upon Sarcina as well as upon the "contaminating" bacillus, then the further question arises as to the constancy or otherwise of the bacteria-ciliate ratio in cultures containing varying initial numbers of infusoria. For if this ratio has the importance which Cutler and Crump's results indicate, then the introduction of equal numbers of Sarcina into each of the cultures containing one, two, or three infusoria would lead to diminished reproductive rate in the cultures containing the greater population of ciliates, and allelocatalytic effect would tend to be counterbalanced by paucity of food supply. So far as one can judge from their description of their technique (which is somewhat indefinite in respect to this point), it appears that Cutler and Crump introduced equal numbers of Sarcina into each culture. The counterbalancing effect of diminishing bacteria-ciliate ratio may therefore have actually contributed in their experiments to mask allelocatalytic effect.

In my own experiments the bacterial inoculum must be very nearly proportional to the number of ciliates introduced, for since the infusoria were washed prior to inoculation into the culture medium, the bacteria introduced with them must have been chiefly those which were closely adherent to the bodies of the ciliates or engulfed within their vacuoles. The bacteria-ciliate ratio must therefore be nearly constant. This raises once more the question, however, whether the allelocatalysis which I have observed is really due to the mutually accelerative action of the ciliates themselves, or to an underlying allelocatalysis of associated bacteria. This possibility occurred to me immediately after obtaining my first positive results, and I repeated my experiments on allelocatalysis, employing media heavily infected with bacteria by exposure to air for 24 hours. I found that allelocatalysis occurred in these "bacterized" infusions no less than in fresh hay infusion. Cutler and Crump, however, have shown that the growth of *Colpidium* is very much more facilitated by the "contaminating bacillus" which was found by them to be associated with this organism than it is by the Sarcina which they employed as a food-organism. This suggests the possibility that *Colpidia* and other ciliates can live only upon particular species of bacteria, and that other bacteria which may be present only affect their growth to the extent that they modify the nutrients available for the food-species of bacteria. If this be the case, then my experiment, upon which I relied to show that allelocatalysis originated in the contiguity of the ciliates and not in that of the associated bacteria, is inconclusive, because the bacteria which develop spontaneously in hay infusion may not be those which the ciliates can employ as food, and the question of the origin of allelocatalytic effect in infusorial cultures still remains unsettled.

Whether the allelocatalytic effect originates with the ciliates, or with associated food-organisms, however, it remains a mutually accelerative effect of contiguous organisms upon their reproductive rates. That is, the contaminating organisms multiply much more than twice as rapidly when the mass of the inoculum is doubled. In fact, if the effect really originates with an associated "contaminating organism," then the estimate of its magnitude which is afforded by the reproductive rates of the infusoria is very inadequate, for, according to the figures supplied by Cutler and Crump, the reproductive rate of the infusoria increases much more slowly than the population density of the food organisms. Nor, if this should eventually be ascertained to be the origin of the allelocatalytic effect which I have observed, does it at all preclude the possibility that allelocatalytic effect also occurs between contiguous pairs of infusoria. To demonstrate its existence, however, would necessitate inoculating the infusoria into a culture-medium already densely inhabited by the food-organism, so that the additional organisms introduced with the ciliates would form an inappreciable proportion of the number already present, but as I am ignorant, at present, of the nature of the food-organisms required by the species of ciliates which I employ, I have been unable to perform this experiment.

If, however, the growth-rate of infusoria is determined by the multiplication of food-organisms which are associated with them, then it is curious that Cutler and Crump should have failed to ascertain any effect of washing the ciliates upon their reproductive rate, more especially since they have suggested that the low maximal populations of ciliates obtained by Peters were due to the absence of any but minimal populations of the contaminating food-organism. It is also peculiar that they have failed to find any favourable influence upon the reproductive-rate of the ciliates, of reducing the volume of the culture-medium into which the inoculum is introduced. Both of these effects would appear to be indicated by their own results. Their failure to obtain such effects suggests that *Colpidium* can utilize *Sarcina* as a source of food as well as the "contaminating organism," and that the allelocatalysis of the associated food-organisms, and the above-mentioned effects as well, were masked by the excess of food-organisms initially introduced into the culture with the added emulsion of *Sarcina*.

Finally, in dwelling upon this possibility, it must be stated that all of the data and conclusions concerning allelocatalysis in infusoria which have issued from this laboratory in recent years remain valid, save that, if the above interpretation is correct, they apply to the associated food-organism and not to the infusoria themselves.

The differences of reproductive-rate among different subcultures of infusoria is extremely great, varying with the age, population, and previous history of the parent-culture, as well as with temperature and the character and volume of the culture-medium employed. For this reason, in the study of allelocatalytic effect, I have always compared the reproductive rates of single individuals and of pairs of individuals, respectively, which are derived from the same parent-culture. Provided care is taken not to consume too much time in abstracting the individuals for isolation (cf. part 3 of this article), the reproductive rates of different individuals isolated from the same parent-culture are almost always identical. Cutler and Crump, however, compare and average the reproductive rates of different cultures containing one, two, or three individuals respectively, which are derived from different parent-cultures of the same age. This leads, naturally, to a great range of variability among the reproductive rates obtained. I pointed out in a previous article, that their (unwashed) single-cell cultures displayed a progressively *diminishing* reproductive rate with diminishing volume of the culture-fluid, suggesting the presence of some inhibiting substance derived from the parent-culture medium. Cutler and Crump maintained, in reply, that the variations of reproductive rate lay within the normal range of variability, and that their progressive augmentation in the order of increasing volumes of culture medium was accidental. In my experience, employing the technique which I have described, they greatly exceed the normal range of variation of reproductive rates in subcultures (derived, that is, from a single parent-culture). Obviously, in seeking for the presence or absence of substances affecting reproductive rate, it would be advisable to employ cultures which, in the absence of the factors sought, might be relied upon to display uniform reproductive rates.

Attention must also be drawn to the fact that allelocatalysis is displayed only between pairs of *living* cells. I have repeatedly investigated the effect of dead infusoria upon the reproduction of living infusoria introduced into the medium containing them. In the volume of medium usually employed for subcultures (0·08 to 0·10 cc.) a single dead *Enchelys* (killed by heating to 40°C) has no effect whatever upon the multiplication of a living individual inoculated into the medium. A second *living* individual, on the contrary (provided that the infusoria have been washed prior to their inoculation into the medium) may accelerate the reproductive rate of each of them to such an extent as to quadruple or even multiply sixteen times the population of the culture at the end of 24 hours Robertson, 14, 16).

It follows from this that if the conditions of inoculation or the character of the medium are such as to lead to an extensive mortality among the individuals which form the subcultures, allelocatalytic effect must be obscured, and that if the mortality attains to 50% of the individuals isolated, allelocatalytic effect must fail altogether to be displayed, because, in the average culture, one of the two individuals inoculated together must die, and the final effect must be the same as that obtained in cultures of single individuals which chance to survive. Unless the cultures were continuously watched in order to observe the multiplication of each of the individuals isolated, comparison of the end-results, coupled with rejection of those cultures which failed to reproduce at all, would obviously lead to the conclusion that allelocatalytic effect does not occur.

So far as the failure of Peskett to observe allelocatalysis in cultures of yeast is concerned, this is all the comment that I propose to make, in view of his admission that the mortality in his cultures ranged from 28% to 50% (10). Whether allelocatalysis occurs in cultures of yeast or not, I cannot say, for I have no experience in the culture of yeasts, but it is perfectly evident that allelocatalysis can never be demonstrated in cultures of any cells among which the mortality may attain 50%. Even one-half of this rate of mortality must obviously mask the effect to a very considerable extent.

It is possible that this factor has also played an important part in masking allelocatalytic effect in Cutler and Crump's experiments upon reproductive rate in *Colpidium*, for they have admitted that in the media which they employ a considerable mortality is common among subcultured individuals (3), and they were careful to point out in one of their earlier papers (4) that "where only one animal is present any growth must be due to that animal, but where two are introduced there is always the possibility that one may have died," but in their recent paper on this subject (6) this possibility is not recalled.

Furthermore, death may occur in these media, not only among the animals originally inoculated into the medium, but also, as Cutler and Crump have themselves shown, among the animals which are produced in the subculture itself, so that the numbers of living animals present after 24 or 48 hours may afford a very misleading estimate of the number actually produced. In my own experiments I have also, in certain media and under certain conditions, observed deaths among the inoculated individuals, and, as will be shown below, diminution in the population of fairly heavily populated cultures. But experiments upon allelocatalysis have never been undertaken excepting under conditions permitting reproduction of all of the subcultured individuals, a fact which was established by hourly examination of the cultures. The origin of the discrepancy between my results and those of Cutler and Crump may therefore be found to depend ultimately upon the discovery of the origin of the deficient and fluctuating viability of cultures in the artificial media employed by these authors, and it was with this object in view that the investigations were undertaken which are about to be described.

2. *Factors influencing the viability of cultures of Infusoria.*

One of the most important factors in determining the viability of subcultures of *Enchelys* is, as stated in earlier papers, the temperature at which the culture is maintained. Subcultures into hay infusion, adjusted to an optimum P_H, almost always die at temperatures above 30°C. The more numerous the inoculum the more chance there is of survival and multiplication of some of the individuals forming the subculture, but the numbers always decrease for some time after subculturing. During the same period the parent-culture remains maximally populated, so that density of infusorial population forms in itself a protecting agent against the adverse effect of high temperature.

The laboratory temperature in South Australia not infrequently rises above 30°C. during the summer, and since it was found impossible to obtain uniform reproductive rates in subcultures in the neighbourhood of this temperature, work upon allelocatalysis has always been discontinued in this laboratory during the summer months. The cultures were maintained, however, by mass-inoculation at frequent intervals, and various endeavours have been made to ascertain the cause of the high mortality in cultures at these temperatures as well as means of overcoming it.

In the course of these investigations two means were found of ensuring the viability of subcultures, provided that the temperature at which they were maintained did not too greatly exceed the limit of 30°C. One consisted in precooling the parent-culture to 4°C. for 24 hours preceding the abstraction of individuals to form the subculture. The age of the parent-culture has a great influence upon the viability of subcultures. Subcultures from very old parent-cultures frequently die, even at temperatures of 20°C. or less. At higher temperatures the infusoria multiply more rapidly, so that the effective physiological age of the cultures at a given number of days is greater at a higher than it would be at a lower temperature. It would seem possible that the cooling of the parent-culture, by slowing or arresting multiplication, practically maintains its physiological age at that of the preceding day. That this is not the sole cause of the greater viability of subcultures from pre-cooled parent-cultures is shown by the fact that these cultures may frequently survive when subcultures made the preceding day from the same parent-culture uniformly failed to survive.

Another way of promoting the viability of subcultures proved to be that of allowing the culture fluid to stand, at room temperature, for 24 hours before subculturing into it. As stated in a former paper, the reproductive rates of infusoria inoculated into this "bacterized" culture fluid are always greater than the reproductive rates of infusoria inoculated from the same parent-culture into unbacterized culture fluid. Not only is reproductive rate increased, however, but also viability. In warm weather it became our custom, when subculturing, always to keep the unused excess of culture fluid until the following day. If the subcultures had failed, then fresh subcultures into the same culture fluid 24 hours after its preparation almost invariably succeeded. That this is due to an alteration of the culture fluid and not of the parent-culture during the interim is shown by the fact that if subcultures are simultaneously inoculated from the same parent-culture into freshly prepared culture medium and medium which is 24 hours old, the latter survive, while the former do not. On the other hand, culture fluid incubated at higher temperatures (37·5°C.) for 24 hours is toxic, and subcultures will not survive in it.

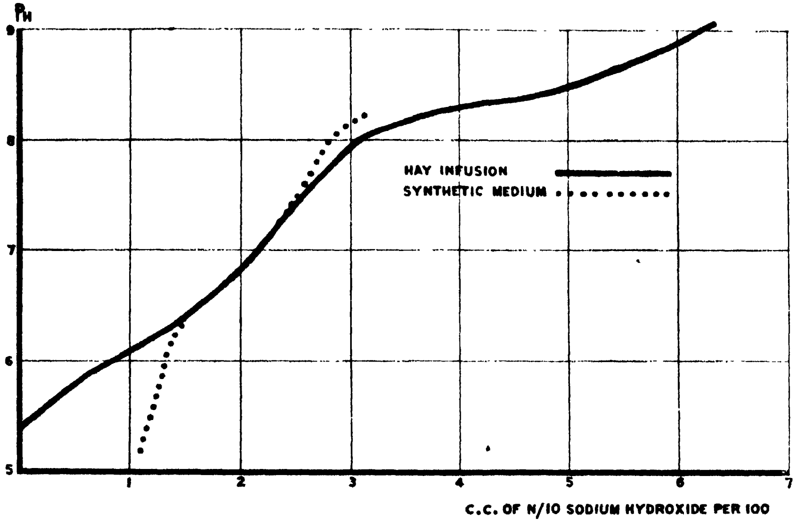

Figure 1. Showing the effect of added acid or alkali upon the P_H of hay infusion and that of synthetic culture medium. To facilitate comparison the titration-curve of the synthetic medium has been shifted so that the two curves coincide at neutrality ($P_H = 7{\cdot}20$). Reactions more acid than this, in the case of synthetic medium, were actually procured by addition of acid equivalent to the alkali indicated on the base-line.

During the course of experiments on the effect of various dyes upon the reproduction of infusoria, it was found that densely populated cultures of *Enchelys* became alkaline, since phenolphthaleon added to the culture fluid, in amounts insufficient to be toxic, turned red as the cultures became heavily populated, indicating a P_H of at least 8·3. Since this alkalinity (also observed by Cutler and Crump) (3) is probably attributable to the metabolic activities of the bacteria contained in the cultures, it appeared possible that the favourable effect of allowing the culture fluid to stand for 24 hours before employing it might arise from an alteration of its P_H. Accordingly, it became of importance to determine the changes of P_H which infusorial cultures undergo with age.

As a preliminary to this investigation freshly prepared hay infusion, obtained by heating 5 grms. of chopped oaten hay in 100 cc. of distilled water until it just begins to boil, transferring to a boiling water bath for one hour, filtering, and making up the volume of the filtrate, if necessary, to 75 cc., and finally aerating with a stream of filtered air for one hour, was titrated against acid and alkali, and the resultant P_H values were determined electrometrically. I am indebted to Mr. Hedley R. Marston, of this department, for carrying out these titrations, the results of which are graphically displayed in figure 1.

It is at once evident from inspection of these graphs that the synthetic medium is much less efficiently buffered, both on the alkaline and on the acid side, but especially on the latter, than the hay infusion. It is interesting to note that hay infusion is especially well buffered in the neighbourhood of P_H = 8 to 8·5, which is the stable final condition of a mature culture of *Enchelys*.

TABLE 1.

Hay Infusion.		Synthetic Medium.[2]	
C.cm. of Alkali added per 100 cc. of Medium.	P_H of Mixture.	C.cm. of Acid or Alkali added per 100 cc. of Medium.	P_H of Mixture.
0·00 cc. $\frac{N}{10}$ NaOH	5·41	1·20 cc. $\frac{N}{10}$ HCl.	5·17
0·44 " "	5·75	1·00 " "	5·92
0·90 " "	6·04	0·80 " "	6·35
1·36 " "	6·32	0·60 " "	6·56
1·81 " "	6·66	0·40 " "	6·78
2·26 " "	7·15	0·24 " "	6·93
2·71 " "	7·66	0·08 " "	7·12
3·16 " "	8·05	No addition	7·22
3·62 " "	8·22	0·16 cc. $\frac{N}{10}$ NaOH	7·30
4·07 " "	8·30	0·32 " "	7·69
4·52 " "	8·39	0·48 " "	7·98
4·97 " "	8·52	0·80 " "	8·20
5·42 " "	8·66		
5·90 " "	8·88		
6·33 " "	9·08		

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[2] The composition of this medium was as follows (Cutler and Crump, 3):

Na_2HPO_4	0·001%	$MgSO_4, 7H_2O$	0·0001%
Ammonium Lactate	0·01%	$CaCl_2$	0·002%
KCl.	0·03%	Glucose	0·04%
NH_4Cl.	0·03%		

The initial reaction of the mixture largely depends upon that of the ammonium lactate. In preparing the medium for the above determinations the ammonium lactate was introduced by mixing standardized solutions of lactic acid and ammonia in the proportions found necessary to secure a reaction of the mixture corresponding to P_H = 7·2.

These data, which are enumerated in Table 1, enable us to compute the initial P_H of hay infusion to which definite proportions of alkali have been added. In order to ascertain the effect of initial P_H upon the viability, duration of lag, and reproductive rates of subcultures, 10 cc. samples of aerated hay infusion were mixed with 0·1, 0·2, 0·3, 0·4, and 0·5 cc of $\frac{N}{10}$ NaOH solution respectively, corresponding to initial P_H's of 6·1, 6·8, 7·9, 8·3, and 8·5. Subcultures were prepared in each of these media from the following parent-cultures: 182A (seven days old), 183A (three days old, kept for the past 24 hours at 4°C.), and 185A (three days old, kept at room temperature throughout). The room temperature was 22°C. A small counted number of individuals was inoculated into each subculture, and no attempt was made to wash them, since all that it was desired to ascertain was the presence or absence of toxicity of the media. The following were the results obtained:

TABLE 2.

Subculture. Number.	Parent-culture.	P_H of Medium.	Initial No. in. Culture.	No. Inhabiting Subcultures after 18 hrs.	19 hrs.	20 hrs.	21 hrs.	24 hrs.	42 hrs.
197A	7 days old	6·1	9	1	2	2	2	4	73
198A	,, ...	6·8	8	6	6	7	11	17	358
199A	,, ...	7·9	9	4	4	4	4	8	250
200A	,, ...	8·3	10	11	13	15	16	25	502
201A	,, ...	8·5	10	4	4	4	8	14	231
202A	3 days old, pre-cooled	6·1	7	14	—	—	18	33	—
203A	,, ,,	6·8	13	41	—	—	76	138	—
204A	,, ,,	7·9	9	43	—	—	67	91	—
205A	,, ,,	8·3	9	40	—	—	88	139	—
206A	,, ,,	8·5	9	39	—	—	67	121	—
207A	3 days old	6·1	14	21	—	—	39	58	—
208A	,, ...	6·8	12	52	—	—	95	163	—
209A	,, ...	7·9	10	38	—	—	56	112	—
210A	,, ...	8·3	9	34	—	—	56	113	—
211A	,, ...	8·5	9	31	—	—	52	95	—

It is evident that the duration of lag, which in subcultures from the seven-day culture lay between 18 and 20 hours, is not appreciably affected by P_H within the range employed. Viability in the most acid culture ($P_H = 6·1$) is diminished, and perhaps for this reason the 24 hours reproductive rate is also diminished. We may infer that an initial P_H in the neighbourhood of 6·1 is definitely deleterious to the organisms, and that in cultures from old parent-cultures the optimum initial P_H is in the neighbourhood of 8·3, the final P_H, that is, of an adult culture. Subcultures from younger parent-cultures, however, maintain an approximately uniform reproductive rate in subcultures in which the initial P_H varies between 6·8 and 8·5.

Having thus established the lower limit of toleration of *Enchelys* for variation of initial P_H in the medium, an experiment was undertaken to ascertain the variation of P_H and bacterial and infusorial populations of cultures during the process of multiplication. The estimations of P_H were carried out by Mr. Hedley R. Marston, and the bacterial counts by Mr. F. Draper, to both of whom I am greatly indebted for their assistance.

The parent-culture was a mass-culture 500 cc. in volume, which had originally been inoculated with 40 cc. of a thickly inhabited culture, and was 18 days old. This was spread in a layer about 0·5 cm. thick over an area of about 1,000 sq. cm., forming the bottom of a glass bowl 18 cm. deep, 26 cm. in diameter at the bottom and 36 cm. at the top. This was covered by a glass electric light reflector, which rested upon the edge of the bowl, and was fastened to the bowl by strips of adhesive plaster. The hole at the top of the reflector was plugged with cotton wool. The subcultures were kept in similar containers, and samples were withdrawn from time to time for the determination of P_H and bacterial count through the opening in the top of the reflector.

Although the parent-culture was 18 days old, and the temperature of the laboratory varied between 22·5° and 23·5° C., the population, estimated from the population of diluted samples, was only 100 per 0·1 cc., and contained many dividing individuals. This difference between the densities of population attained in drop-cultures and those attained in like time in mass-cultures was invariably observed. From this it is evident that cessation of reproduction in cultures is not due to age, in itself, or to bacterial multiplication, or to the variation of P_H induced by bacteria, but directly to the previous multiplication of the infusoria themselves. This, occurring as it does but slowly in mass-cultures, continues to occur, in consequence, for a prolonged period. The probability is that in layers 0·5 cm. deep oxygen is the limiting factor in determining rate of development. Multiplication would therefore occur only in the surface layers, while the accelerative (and ultimately retarding) substances produced during division are distributed through the whole mass of the fluid by diffusion, so that the concentration of autocatalyst in the region to which multiplication is confined does not correspond to the extent of multiplication which has occurred, and the cultures therefore age more slowly than drop-cultures in which oxygen is available in excess.

A litre of hay infusion was prepared by heating 67 grms. of chopped oaten hay with 1,333 cc. of distilled water to boiling, and then heating on a boiling water bath for one hour. The mixture was filtered, and the filtrate, when cool, made up to 1,000 cc. with distilled water, and aerated with a strong current of air (filtered through sterile wool) for one hour. The aerated filtrate was divided into two portions of 500 cc. each. One was buffered with 20 cc. of $\frac{N}{10}$ $NaHCO_3$ and the other with 18 cc. of $\frac{N}{10}$ NaOH. The culture fluids were then placed in receptacles as described above, and each of them was inoculated with 20 cc. of parent-culture (without washing). Immediately after inoculation and thorough mixing, three $\frac{1}{10}$ cc. samples were withdrawn from each subculture and placed in the central depressions of sterile slides, which were kept upon pads of wet filter paper in Petri dishes for observation of the increase in infusorial population. At the same time, and at intervals subsequently, larger samples were withdrawn for the determination of P_H and bacterial count. The P_H values were determined electrometrically in a Clarke shaking electrode, the bacterial counts by dilution and spreading measured volumes over the surfaces of sterile agar plates, which were subsequently incubated, and the colonies counted.

The following were the results obtained:

TABLE 3.

Time (Hours)	Infusoria per Individual originally introduced (Average 3 per 0.1 cc.)		P_H		Bacteria per cc.	
	NaOH Culture.	$NaHCO_3$ Culture.	NaOH Culture.	$NaHCO_3$ Culture.	NaOH Culture.	$NaHCO_3$ Culture.
0	1	1	8·65	7·30	0·50 x 10^{6}	0·43 x 10^{6}
2·5	1	1	8·45	7·50	2·00 x 10^{6}	1·89 x 10^{6}
19·0	10·7	11·0	7·15	7·66	47·70 x 10^{6}	49·00 x 10^{6}
26·5	22·9	17·0	6·93	7·34	86·60 x 10^{6}	108·50 x 10^{6}
43·0	23·5	12·3	7·20	7·81	3·30 x 10^{9}	3·80 x 10^{9}
67·0	200 (about)	152 (1 culture only)	7·98	7·98	48·00 x 10^{9}	52·80 x 10^{9}
91·0	—	—	8·10	8·10	9·98 x 10^{12}	10·78 x 10^{12}
115·0	—	—	—	—	101·0 x 10^{16}	123·0 x 10^{16}
139·0	—	—	—	—	66·0 x 10^{18}	83·0 x 10^{18}

It is evident that the bacterial counts in both cultures were the same within the experimental error. A scum formed upon the surface in both cultures on and after the third day. As this presumably represented bacteria and bacterial products not available for the nutrition of the infusoria, the samples withdrawn for counts were taken from below the scum, but, on account of the scum, the cultures could not be mixed before taking the samples.

Despite the similarity in bacterial contents, however, the rate of infusorial reproduction differed considerably in the two cultures. The production of infusoria in both cultures was checked during the second 24 hours, but in the culture buffered with $NaHCO_3$ the number of infusoria actually underwent diminution, so that at 43 hours the infusorial population in the $NaHCO_3$ culture was about half that in the NaOH culture. In the succeeding 24 hours the infusorial population underwent great increase in both cultures. These observations correspond to those reported by Cutler and Crump (3, 5), who observed irregular fluctuations of reproductive rate in their cultures of *Colpidium*, and frequently temporary diminutions of population. It would appear from the above-cited results that these fluctuations do not originate in fluctuations of bacterial population. It must be admitted, however, that the evidence of this is inconclusive, because, in the first place, we are not certain that the type of bacterial population, which is estimated by growth on agar plates, is the type

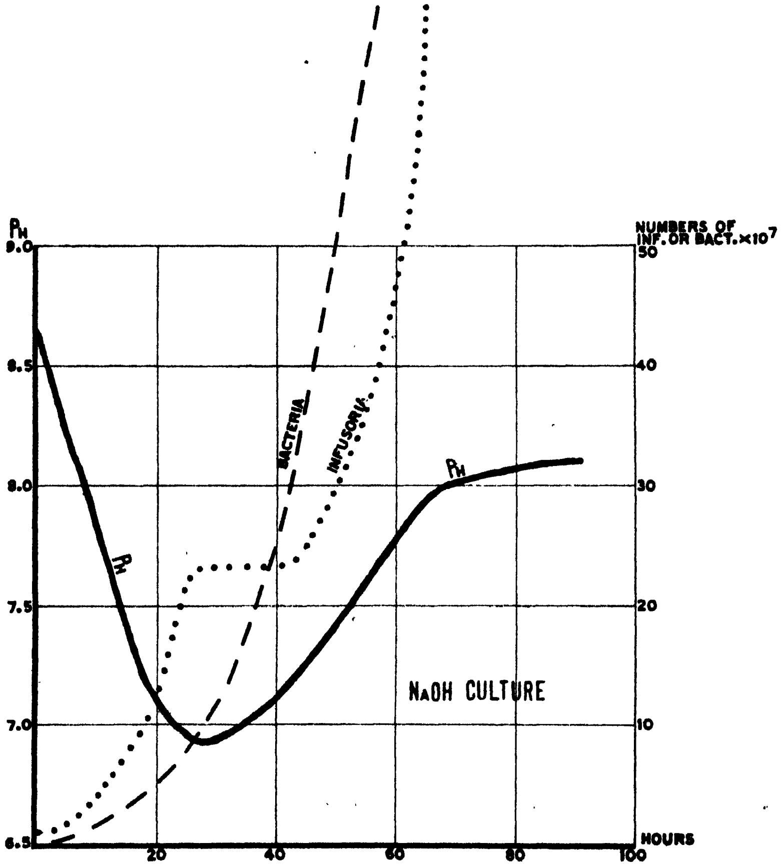

Figure 2. Showing the changes of P_H and bacterial and infusorial populations subsequently to inoculation of hay infusion originally brought to $P_H = 8{\cdot}65$ by addition of NaOH. Temperature 22·5°C. to 23·5°C.

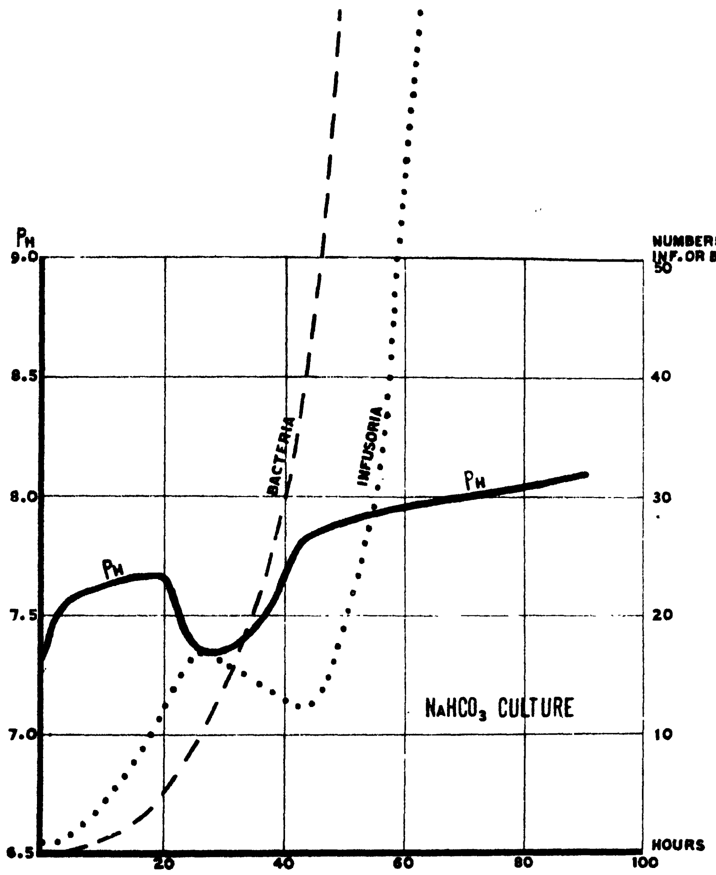

Figure 3. Showing the changes of P_H and bacterial and infusorial populations subsequently to inoculation of hay infusion originally brought to $P_H = 7{\cdot}30$ by addition of $NaHCO_3$. Temperature 22·5°C. to 23·5°C.

upon which the multiplication of *Enchelys* depends, and in the second place, the infusorial population was estimated in drop-cultures, while the bacterial population was estimated in bulk-cultures.[3]

On the other hand, the estimates of the P_H values show that in both cultures the P_H attained a minimum value at 24 hours, coincidently with the check in reproduction of the infusoria (cf. figures 2 and 3). It is curious, however, that the most severe check upon reproduction of the infusoria occurred in the $NaHCO_3$ culture in which the minimum P_H was 7·34, while in the NaOH culture, which suffered less, the P_H fell as low as 6·93. As 16½ hours elapsed before the next P_H determination was made, we cannot be sure that the observed minimum of P_H was the actual minimum, but it is evident that in the $NaHCO_3$ culture in 7½ hours, during which the P_H fell from 7·66 to 7·34, a definite check had been experienced by the infusoria, the effect of which endured for a considerable time, while a fall from 7·15 to 6·93 in the NaOH culture during the same period was less injurious. It may be that the P_H changes in the bulk-cultures did not faithfully reproduce the changes occurring in the drop-cultures, which may have been more rapid or more profound, but, on the other hand, it is possible that in the $NaHCO_3$ culture the CO_2 tension was higher, so that the effect of outside P_H upon the P_H in the cells themselves was greater than it was in the NaOH culture, since the penetrability of cells for CO_2 is greater than their penetrability for other acid radicles (Jacobs, 1920).

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[3] The infusorial population could not be estimated directly in the bulk-cultures, because the presence of the bacterial scum after the second day prevented mixing of the cultures, and the infusoria were not distributed evenly through them, but, as explained above, were concentrated in the surface layers.

Whatever its origin, however, it is evident that in these cultures, whether initially alkaline (8·65) or neutral (7·30), the P_H diminishes either immediately (in the initially alkaline culture) or after a period of increase (in the initially neutral culture), so that in either case there is a phase of relative acidity which culminates at about 24 hours, under the conditions of temperature, etc., which are employed in this experiment. Subsequently to this the P_H increases until it attains a value lying between 8·0 and 8·3, which is the final condition attained in both the cultures, irrespective of the value of the initial P_H. It is in this region also that hay infusion is most effectively buffered (figure 1).

During the phase of relative acidity the conditions are injurious for the infusoria. Obviously, if the culture medium is inadequately buffered in the region between $P_H = 6·0$ and 7·0, the chance of arrest of multiplication, or even death, of the infusoria must be much greater than in media which are more effectively buffered in this region. On the acid side of $P_H = 6·4$, and on the alkaline side of $P_H = 7·8$, the synthetic medium employed by Cutler and Crump is much less effectively buffered than hay infusion. It is therefore a less efficient medium, both for preserving the viability of the organisms and for maintaining the final conditions towards which cultures of maximum population tend.

3. *The Variation among Individuals Inhabiting Cultures of Infusoria.*

It has been shown by Jennings (9) that the individuals inhabiting a culture of *Paramoecia* are not morphologically alike, and that the morphological differences can be perpetuated through a large number of generations.

It is evident from the results obtained by Cutler and Crump with *Colpidia*, and from the results enumerated above, that the individuals inhabiting a culture of infusoria also differ among themselves physiologically, and that these differences are evidenced by differing sensibility to adverse conditions, i.e., in the above experiments, to an unfavourable reaction of the medium. Not all of the individuals which inhabit the culture die when the reaction becomes unfavourable, but only a proportion of them. While in rapidly multiplying cultures, as in the experiments described above, the physiological differences underlying differences of sensibility to toxic agents might conceivably originate in differences of age, it has also been found, both by Cutler and Crump and by the author, that similar partial mortality occurs in adult cultures in which multiplication is no longer taking place.

It has been shown by Peters that similar differences occur in the sensibility of the individuals comprising an infusorial population towards the toxic effects of antiseptics, and he suggests that if similar differences subsist between bacteria, the distribution of their susceptibilities in a frequency-curve would lead to time-relations of disinfection similar to those which characterize a monomolecular chemical reaction (11). He overlooks the fact, however, that the monomolecular law itself originates in a similar chance distribution of instability in the reacting molecules (or else of their situation in space), from which, as the author has shown, the monomolecular law of disinfection may also be deduced (13). The experiments of Peters, therefore, do not reveal the origin of the monomolecular law of disinfection, but merely the impossibility of deciding, from the time-relationships alone, whether the distribution of deaths in time is primarily attributable to the fortuitous distribution of molecules in space or to the fortuitous distribution of susceptibility in the organisms. It is evident, however, that differences of susceptibility do obtain between the individuals which comprise a population of infusoria.

The existence of such differences, capable, moreover, of affecting the multiplicative rate in subcultures, is revealed by the following facts:

If a large number of subcultures are prepared from a single parent-culture, whether the isolated individuals are washed or not, it is very frequently found, on warm days, that the last subcultures display a much higher reproductive rate than the earlier ones. This is due, however, to the greater viability of the later cultures, and not to differences in the reproductive rates of survivors, as the following experiments reveal:

Experiment 1. Nine subcultures taken from a parent-culture 5 days old; the organisms were not washed:

Serial Number of Subculture.	Initial Number of Organisms.	Number contained in Cultures after			
		18 hours.	22 hours.	26 hours.	42 hours.
First	8	2	2	2	6
Ninth	6	6	6	6	13

Experiment 2. Seven subcultures taken from a parent-culture 4 days old; the organisms were not washed:

Serial Number of Subculture.	Initial Number of Organisms.	Number contained in Culture after		
		19 hours.	23 hours.	26 hours.
First	14	5	5	7
Seventh	7	7	10	10

Experiment 3. Eight subcultures taken from a parent-culture 4 days old; the organisms were not washed:

Serial Number of Subculture.	Initial Number of Organisms.	Number contained in Cultures after		
		18 hours.	22 hours.	43 hours.
First	1	0	0	0
Second	1	0	0	0
Eighth	1	1	1	15

Experiment 4. Nine subcultures taken from a parent-culture 3 days old; the organisms were washed once:

Serial Number of Subculture.	Initial Number of Organisms.	Number contained in Cultures after	
		19 hours.	26 hours.
First	4	3	5
Ninth	4	4	10

Experiment 5. Nine subcultures taken from a parent-culture 3 days old; the organisms were not washed:

Serial Number of Subculture.	Initial Number of Organisms.	Number contained in Cultures after 18 hours.
First	26	0
Seventh	29	138
Eighth	31	327
Ninth	25	185

Numerous results of the same character were obtained, but those reported show the distinctive features of the phenomenon. The lag-period is not affected. The reproductive rates of those individuals in the earlier cultures *which chance to survive* are not diminished, but the mortality in the early cultures is much higher than it is in the later cultures. It will be recollected that this is only true, as a rule, in hot weather (temperature 25° to 30°C.), provided that the hay infusion is initially brought to a P_H lying between 7·3 and 8·3 by the addition of bicarbonate or sodium hydroxide in suitable proportions.

In experiments designed to compare the effects of successive modifications of or additions to the culture medium upon the reproductive rate of the organisms, the phenomenon, when it occurred, was very troublesome. Whenever experience showed that it was likely to occur the precaution was taken of culturing into normal hay infusion at the beginning and at the end of each series of subcultures. If the apparent reproductive rates in the first and last subcultures were then found to differ materially, the entire experiment was rejected.

The above-cited results were obtained in experiments which were rejected for this reason.

Another way of overcoming the effect which was frequently adopted was that of diluting a small proportion (0·003 to 0·005 cc.) of the parent-culture with from 20 to 25 times its volume of fresh hay infusion, and isolating all the subcultures from this sample. The subcultures were then almost uniform in their behaviour.

Reviewing the technique of the operations leading to the isolation of individuals to form subcultures, three possible origins of the phenomenon suggested themselves, namely:

(*a*) Exposure to the strong artificial light employed in counting the isolated infusoria, and which was allowed to fall upon the parent-culture during the time occupied in isolating subcultures. At other times the cultures were only exposed to dim diffused daylight.

(*b*) Evaporation from the surface of the culture after removal of the cover of the Petri dish in which it was kept, leading to an increase of osmotic pressure of the parent-culture.

(*c*) Reduction of the volume of the parent-culture by repeated removal of samples. The samples removed were frequently more numerous than the subcultures, because certain samples, containing too many or too few organisms, were always rejected.

Direct experiment showed that (*a*) was not the origin of the effect. A sample was withdrawn from a culture; the individuals in it were counted, and a subculture formed from them. The parent-culture was then exposed to the strong light for an hour, and another sample withdrawn, counted, and subcultured. In every case the viability of the two cultures was the same. It might be suggested that this experiment also ruled out possibility (*b*), since the cover of the Petri dish was removed to allow the light to play directly upon the drop containing the culture. But evaporation would obviously be more rapid the smaller the drop, so that as the size of the drop was reduced by successive withdrawals of samples, the increase in osmotic pressure might possibly be considerably greater than that arising from exposure of the undiminished drop to free evaporation for an hour. It still remained possible, therefore, that the origin of the phenomenon resided in a combination of the factors (*b*) and (*c*).

A number of experiments were carried out, therefore, in which concentrated Ringer solution was added, in small proportion, to the hay infusion, in order to increase its osmotic pressure without diluting it to any important extent. In some experiments the organisms were directly subcultured into these media. In others they were immersed in the hypertonic media for varying periods, and then transferred to normal hay infusion, forming the subcultures.

It was found that *Enchelys* is remarkably resistant to hypertonicity arising from inorganic salts. On first introducing the organisms into these hypertonic media the withdrawal of water was very manifest, and the organisms were compressed laterally into disc-shaped bodies. Within five or ten minutes at the utmost, however, in concentrations of added salts varying from ⅙ the concentration of mammalian Ringer to its full concentration, the organisms regained their normal contour, although, in the highest concentrations (full strength added Ringer) the organisms ultimately died. Cultures in hay infusion containing half-strength added Ringer ($= \frac{M}{12}$ sodium-chloride) sometimes survived without diminution of number for 48 hours, but no divisions occurred. In lower concentrations of added Ringer the organisms not only survived, but underwent reproduction at a rate only slightly less than normal. The following is an illustrative experiment:

Parent-culture 3 days old; temperature 25°C

Culture No.	Concentration of added Ringer.	Number of Infusoria produced per initial individual after 18 hours.
280A	None	18
281A	None	22
282A	M/96	15
283A	M/48	16
284A	M/24	15
285A	M/12	0
286A	M/6	0
287A	None	22
288A	None	18

No improvement of reproductive rate or viability was observed in any of the hyptertonic media. The effect of evaporation of the parent-culture during the processes of subculturing, however, would be to expose the organisms for a while to a hypertonic medium, followed by return to a normal medium, now hyptonic in comparison with the medium to which they have become adapted. Experimental imitation of these conditions, however, yielded uniformly negative results. In no case was the viability of subcultures improved; on the contrary, if the hyptertonic media contained from $\frac{M}{12}$ to $\frac{M}{6}$ added Ringer, exposure for 5 or 10 minutes to these concentrations followed by return to normal hay infusion, usually resulted in death of all the individuals in the subculture. Exposure of similar duration to less hypertonic solutions had no effect whatever upon the reproductive rates or viability of the organisms.

It appears, therefore, that we must attribute the superior viability of late samples withdrawn from the parent-culture primarily to factor (*c*) enumerated above.

Low and slow-swimming organisms, usually of a more spherical outline than those which swim actively and high in the drop, are difficult to withdraw from a drop of 0·1 cc. volume in a sample of 0·003 to 0·005 cc. An extreme example of this is afforded by the cysts of the large infusorian employed in experiments reported in previous articles (17, 18).[4] These lie at the bottom of the drop, and a sample withdrawn from it contains, as a rule, no cysts at all, or far less than the due proportion of cysts. The cysts themselves are sticky, and adhere somewhat to the bottom of the slide, but the statement remains true, even when the cysts are detached by rubbing the bottom of the slide with a pointed glass rod. Moreover, for a considerable period prior to actual encystment these organisms lie quiet at the bottom of the drop, and these are equally difficult to withdraw in the first samples. Only when the drop becomes so diminished in volume that these low-swimming forms are densely crowded do they appear in their due proportion in the samples withdrawn.

Enchelys similarly, but to a much less degree, varies in its form and habit of swimming, and low-swimming forms are usually of the well-nourished, spherical type described in a previous communication (14). For this reason they are ill-represented in samples withdrawn from a large drop, and it is probable that the greater viability of the individuals withdrawn in the later samples is due to the contraction of the dimensions of the drop and the greater proportion of low-swimming individuals which are withdrawn in the sample. Evidently, in an adult culture, such individuals do not differ from the high-swimming type in reproductive rate or lag, but they differ from them in the superiority of their resistance to the adverse conditions which may arise during the first 24 hours in newly inoculated cultures.

SUMMARY.

1. Allelocatalysis (mutual acceleration of reproduction by contiguous cells) is displayed only between pairs of *living* infusoria. It follows from this that if the conditions of inoculation or the character of the medium are such as to lead to an extensive mortality among the individuals which form the cultures, allelocatalytic effect must be obscured, and that if the mortality attains to 50% it must fail altogether to be displayed.

2. Subcultures of infusoria *(Enchelys)* which are isolated into hay infusion almost always die at temperatures in the neighbourhood of 30°C. Two means have been found of ensuring the viability of these subcultures, provided that the temperature at which they are maintained does not too greatly exceed the limit of 30°C. One consists in pre-cooling the parent-culture to 4°C. for 24 hours preceding the abstraction of individuals to form the subculture. The other consists in allowing the culture fluid to stand for 24 hours at room temperature before subculturing into it.

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[4] This organism was submitted for identification to Dr. J. S. Dunkerly, who very kindly informs me that it is *Colpoda cucullus* (Miller).

3. Hay infusion and the synthetic culture medium employed by Cutler and Crump have been titrated electrometrically. It was found that whereas they are equally buffered between the P_H values of 6·4 and 7·8, the synthetic medium is much less effectively buffered against excess of acid or alkali than hay infusion for P_H values below or above these limits.

4. The changes in P_H, bacterial count, and infusorial population of subcultures at 23°C. have been investigated. It is found that the P_H of subcultures, whether these are initially alkaline or initially neutral, at first falls and later rises, ultimately reaching a stable value in the neighbourhood of 8·0 to 8·3. During the period of relative acidity the infusorial population remains stationary or diminishes. Multiplication is resumed as the reaction becomes more alkaline. The multiplication of bacteria, on the contrary, is unaffected by these changes in the reaction of the medium.

5. The members of a thickly-inhabited infusorial population differ among themselves in their viability when transferred to freshly prepared culture medium. The low-swimming individuals of more spherical outline are most resistant to the adverse conditions which may arise at some time during the first 24 hours in subcultures. The lag-period and reproductive rate do not vary to nearly so great an extent among different individuals as their viability after isolation.

6. *Enchelys* is remarkably resistant to hypertonicity arising from inorganic salts. Cultures in hay infusion containing added Ringer solution equivalent in concentration to $\frac{M}{12}$ sodium-chloride sometimes survive without diminution in number for over 48 hours, but without undergoing division. Viability is not improved by either brief or prolonged exposure to hypertonic media. Brief exposure to hypertonic media does not affect the subsequent duration of lag or the reproductive rate, provided the hypertonicity ($\frac{M}{12}$ added Ringer or over) is not sufficient to bring about the death of the organisms which are exposed to it.

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A FURTHER NOTE ON THE STRUCTURE OF THE NEURONE JUNCTIONS OF THE SPINAL CORD

by

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In previous papers in this journal I have pointed out that there is no unquestionable evidence available for the conception of neurones terminating upon the body of nerve cells in the spinal cord by ramifying synapses which embrace the body of the nerve cell. On the contrary, it was concluded that the neurone junctions take place via the cell dendrites. The collaterals, entering the grey matter from higher or lower levels of the cord undergo extensive branching in the grey matter, and these branches, travelling in various directions within the grey matter, enter, either singly or in conjunction with the branches of other collaterals, one or other of the numerous dendrites of the nerve cells.

It has hitherto been impossible to bear this statement out by means of direct observation, since it is practically certain that the nerve cells which form part of a reflex arc will not be included in their entirety within a section of the cord of the thinness necessary for microscopic observation. The evidence on which the statement is based is as follows:

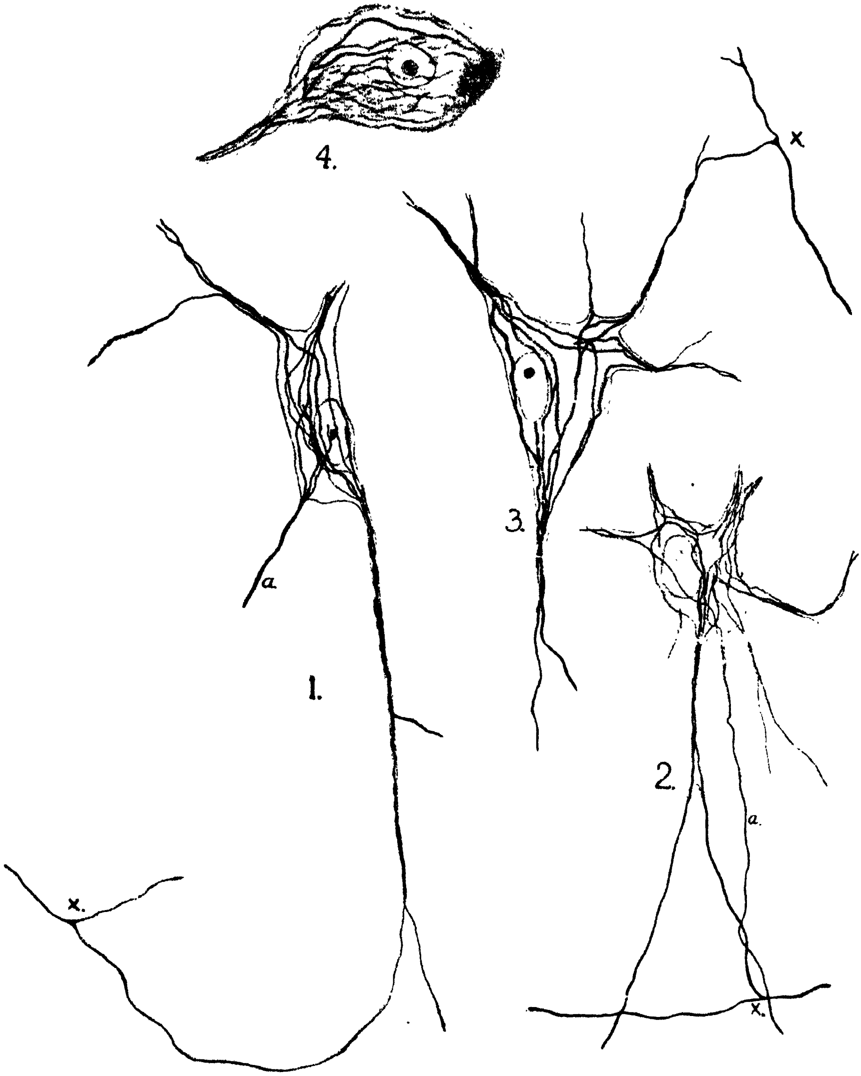
(*a*) The neurofibrils of the nerve cells leave the body of the nerve cells, and, having entered the dendrites, emerge from these, and travel for long distances as naked neurofibrils in the grey matter of the cord. No real termination of these fibrils can be revealed after they emerge from the nerve cells, since their ending is only an apparent one, due to the fact that they are cut short as they leave the plane of the section.

(*b*) In preparations made by the reduced silver method, in which apparently the nerve fibres have stained almost in their totality, no indication of branching synapses can be detected (10). This might be taken as valueless negative evidence, since it might be urged that the branching "synapse" had a different staining reaction from the rest of the nerve fibre, so that the terminal part of the fibre would not be rendered visible. This objection is disproved by the fact that it would reveal an obvious discontinuity of the fibres at the points where the staining had ceased; but such discontinuity, as already stated, does not exist.

(*c*) When the collaterals are traced from the white into the grey matter they are found to undergo branching; but these branches cannot be shown to have any relation to the nerve cells of the grey matter, for sooner or later they always emerge from the plane of the section. But these nerve fibres have precisely the same appearance as the naked neurofibrils that emerge from the cell dendrites, and since these latter also cannot be shown to have any ending, and since no branching synapses have been demonstrated, it is concluded that the collaterals are one and the same thing as the naked neurofibrils that leave the nerve cells through their dendrites.

Since making these observations it occurred to me that there was a simple means available for recognizing collaterals within the grey matter, quite apart from tracing continuity between them and a definite axon. This depends on the fact that a collateral usually undergoes a certain amount of branching within the grey matter; sometimes, indeed, the branching is considerable. A branching collateral may usually be recognized by the fact that at the point of branching there is a small triangular piece of protoplasm, and this piece of protoplasm is so located, also, that it enables us to recognize the direction in which the fibre is travelling. Sometimes branching of neurones occurs without such a distinguishing feature, but these fibres have never been used as a means of recognizing collaterals in this investigation, since a certain amount of uncertainty always attaches to their identification in cases where we are dealing with two fibres that run one above the other for a considerable distance, and then suddenly separate. The object of the investigation was to find collaterals, recognizable by this feature, which, even if they could not be traced outwards in the white matter, yet could be traced into the cell dendrites. The making of the observations is attended by a great deal of difficulty. Thick and fairly completely stained sections are necessary; when the point of branching of a collateral is detected the two branches are followed in the direction in which the fibre is running. Very frequently it is impossible to trace these branches far with certainty, since they become confused with other fibres, and it is practically impossible to follow them individually with certainty; but much more frequently, sometimes after they have travelled a short distance, at other times after one has successfully followed them for very long distances, they will gradually or suddenly bend, and move out of the plane of the section. In fact, hundreds of attempts to follow these collaterals are but awarded with one or other of these exasperating results. But occasionally I have actually succeeded in tracing a branch from these collaterals into the cell dendrite.

Three examples of this are shown in figures 1, 2, and 3, taken from the spinal cord of a young rabbit, and prepared by Cajal's reduced silver method. When examining the branching collaterals, two types of branching may be distinguished: there are those cases where the fibre divides into two branches of approximately equal thickness, or there are cases where one of the branches is of extreme delicacy, with a diameter even smaller than that of most neurofibrils;



REFERENCE TO PLATE.

Figures 1 and 2. Two anterior horn cells from rabbit (Cajal reduced silver method). A collateral undergoes branching at *X*, and one of the branches penetrates the dendrite of the anterior horn cell and becomes identical with one of its neurofibrils. a - - - axon.

Figure 3. Posterior horn cell, showing the same.

Figure 4. A nerve cell from spinal cord of unborn rabbit, unfixed, and bathed in amniotic fluid. An attempt has been made to represent the individual fibrils as accurately as possible.

and it is a particularly difficult matter to follow this latter type of fibril for any considerable distance. In figure 1 is shown a case where a very delicate collateral divides into two extremely delicate branches, and one of these enters a dendrite of an adjacent nerve cell; the other branch emerges from the plane of the section. In figure 2 is shown a collateral giving off a considerably thicker branch to an anterior horn cell. Figure 3 shows a similar case from a cell of the posterior horn, closely adjacent to the substance of Rolando. The fact that only a small number of such cases has been found, while on the other hand every nerve cell that is at all well stained shows numerous neurofibrils emerging naked from the dendrites, might be taken to mean that little importance can be attached to this mode of neurone junction. The criticism would be unjust, for in the majority of collaterals that one observes, the manner of branching is such that it would be impossible to reveal their actual junction with the nerve cells.

From this direct observation we are therefore able to conclude that the predominant type of neurone junction in the spinal cord is a direct junction via the cell dendrites. Whether a second type of neurone junction in the form of branching synapses applied to the cell body occurs cannot be stated at present. The reduced silver method fails to reveal them. The terminal "buttons" which Cajal has described by this method cannot be accepted without further evidence; they have never been shown to be the terminations of undoubted nerve fibres Similar effects may often be obtained, especially in older animals, at the surface of nerve cells which have undergone marked shrinkage, and the fibrils appear to represent coagulated intercellular matrix drawn into fibrils during the shrinking of the cell. On the other hand the possibility has not been excluded that they may be terminations of neuroglia fibres. Golgi observed many years ago that neuroglia fibres may end in this manner upon blood vessels, and Cajal gives many illustrations of the same. In addition to the observations recorded in a previous paper (9), I have made numerous examinations of spinal cords stained intravitam with methylene blue, but have never observed a true synapse. It was by this method that Cajal believed he had rendered such a synapse visible. The observation is, however, open to question. Cajal remarks (5, p. 312), that while the Weigert-Pal and Ehrlich methods are capable of revealing the collaterals in the region where these branch off from the longitudinal fibres of the white matter, these methods are quite unsuitable for tracing collaterals far into the grey matter of the cord. Nevertheless, it is with this admittedly defective technique that the most convincing example of a synapse has been described. Until the observation can be confirmed by others it cannot be accepted. In numerous methylene blue preparations, prepared by Cajal's diffusion method, or by Dogiel's immersion process, I have failed to obtain any indication of them. I find, on the contrary, that the surface of the nerve cells and the intercellular matrix of the cord (especially the latter) may undergo an extraordinary amount of "frothing" and disorganization during the preparation of the material; this is in the main probably due to the fact that no true fixation of the tissue occurs. While methylene blue is an excellent stain for such peripheral plexuses as the Auerbach plexus, it cannot at present be so satisfactorily applied to the central nervous system. Much seems to depend on the type of methylene blue employed. I have used the "med. pur." and the "Grübler nach Ehrlich" samples, as well as several other less known ones, but while these give excellent results with the intestinal plexus, they have been practically useless for the spinal cord. Satisfactory staining of the nerve cells is usually easily obtained, but the finer nerve fibres do not react at all well; but even in cases where the fine nerve fibres of the grey matter have been partially stained, no trace of synapses can be seen. On the other hand, the artefacts obtained by this method are so extensive that it is often possible to see almost anything into the preparations.

By means of the Golgi method Cajal has observed and figured numerous instances of collaterals breaking up into small bunches of delicate fibrils. Most of the illustrations to chapter XI of his book would serve as examples. None of these cases, however, affect the point at issue; that the collaterals undergo extensive branching is not, of course, denied, but that these bunches of fine fibres are terminal synapses does not by any means follow. None of the branches are actually shown terminating upon the nerve cells, though they are certainly mostly in the neighbourhood of the nerve cells. Even this does not always hold true; for instance, in figure 111 of his book many of the collaterals appear to terminate in the region of the substance of Rolando, which is devoid of nerve cells, and even in the posterior horn one does not find by any means as numerous nerve cells as the number of nerve terminations would lead one to expect.

The conclusion that we seem justified in drawing from these observations is that the majority of neurone junctions in the cord occur by collaterals directly penetrating the dendrites of the nerve cells, and becoming continuous with their neurofibrils, and that the evidence for the existence of synapses is inadequate. Even if cases of fibrils terminating on the body of nerve cells be discovered in the spinal cord, the contact theory will still be doubted. Thus F. M. Ballantyne (2), using the lung-fish (Lepidosiren) as material, has recently discovered and figured nerve fibres which undergo a small amount of branching near the body of the nerve cells, and having penetrated the body of the nerve cell, become identical with its neurofibrils.

These observations, moreover, cast doubt upon the theory of contact rather than of continuity, as applied to the remainder of the central nervous system. Those cases in which definite discontinuity has been observed are largely deprived of significance, in that discontinuity may be an artificial result of shrinkage of the nerve tissue occurring during its preparation. Attention may be called to the fact that in one case of nerve termination, namely, the ending of a motor nerve upon a "striated" muscle fibre, we have continuity and not contact merely. Such a nerve termination does not seem to differ essentially from the termination of one neurone upon another: embryologically they are similar; if the nerve fibre in either case be sectioned degeneration does not primarily proceed beyond the ending of the nerve fibre, and degeneration of the next nerve cell, or of the muscle fibre, is a secondary and much delayed event, due to disuse apparently, and not to its isolation. Other points of similarity in the two are a marked slowing of the rate of conduction at the junction and a readiness to undergo fatigue. In this case of motor nerve endings upon "striated" muscle fibre, a case for which a fairly satisfactory technique has been worked out, contact rather than continuity has frequently been described. Nevertheless, the recent work of Boeke (3) shows beyond all question that true continuity does occur.

Histological research leads us to the conclusion that the neurofibril, rather than the neurone, is the elementary conducting unit of the nervous system. Against this conception has frequently been raised the objection that the neurofibril cannot be observed in living nerve cells, and is merely a post-mortem coagulation product. This denial of their existence, a view maintained by F. W. Mott and by Marinesco, loses its significance when we recollect that uncoloured structures will only be visible within nerve cells if their refractive index differs markedly from that of the cytoplasmic fluid which bathes them; a microscope slide, standing in a beaker of water, is invisible except at its rough edges (where the light is irregularly reflected), but it would not be justifiable to deny the presence of the glass slide merely because it cannot be seen. Boeke, on the contrary, remarks that in the nerve cells of the leech, treatment of the nerve cells with very dilute methylene blue, which does not coagulate the protoplasm, results in the staining of these neurofibrils (4). Even if methylene blue were toxic for the nerve cell, it would scarcely be expected to bring about the same coagulation picture as such a substance as alcohol, which is used in the Cajal reduced silver technique. Nevertheless, the two methods give similar results.

In contradiction to the view that the neurofibrils are artefacts, I may remark that I have frequently observed a faint fibrillary structure in unfixed and apparently living nerve cells from young rabbits, bathed in their own body fluids. That the fibrils cannot be seen with a clearness in any way approaching that of the fixed and stained preparation is true, but the fact that a fibrillation may at times be detected is itself strong evidence that the neurofibrillae are not mere fixation products. Such a case of fibrils within an unfixed nerve cell is shown in figure 4. It must be admitted, at the same time, that usually no trace of fibrils is to be seen within the living nerve cells.

In addition to the observation of Boeke on the nerve cells of the leech, it may be recalled that Max Schultze observed a fibrillary structure in living nerve cells bathed in serum, and both Apathy (1), and especially Göthlin (6), examining the living nerve cells of various vertebrates and invertebrates by means of polarized light, have established the existence of neurofibrils in unfixed nerve cells. Similarly, Howard (7) records the presence of neurofibrils in the fresh unfixed visual cells of vertebrates. These various observations seem to show that a system of fibrils, even if indistinct, can be revealed within the body of unfixed living nerve cells under favourable conditions.

It may at the same time be pointed out that if a portion of fresh spinal cord of a young rabbit be examined in serum, while it is true that the neurofibrils are rarely (and then only indistinctly) visible, so also it is usually impossible to detect any trace of the enormous number of extremely delicate collaterals which the reduced silver and Golgi methods demonstrate in the grey matter. It is of further interest to note that in not a single nerve cell examined fresh in serum is any trace visible of the numerous synapses that are supposed to be present on its surface. The microphotographs of Marinesco (8) also reveal no trace of them. It does not therefore seem justifiable to deny the existence of neurofibrils in living nerve cells, when we certainly have good evidence for their existence in such cells, and at the same time uphold the thory of numerous synapses embracing the body of the nerve cells, when the evidence for their presence is so very scanty.

SUMMARY.

1. Collaterals are recognizable within the grey matter of the spinal cord by the fact that they undergo branching, the point of branching being marked by a small triangular piece of protoplasm.

2. Most of the collaterals thus identified, when followed along their length, emerge from the plane of the section, or become confused with other fibres.

3. But occasionally it is possible to trace these fibrils right into the dendrites. This is taken as direct evidence in support of the view previously put forward on indirect evidence, that there is a direct neurone continuity in the spinal cord, the collaterals entering the nerve cells via the cell dendrites.

4. No evidence for the existence of synapses branching on the outside of the nerve cells has yet been found.

5. In support of the view that the neurofibrils are genuine cell structures, it is pointed out that under certain circumstances they may be seen in the unfixed nerve cell.

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THE SUPPOSED DISTINCTION OF AUSTRALIAN ABORIGINALS INTO "STRAIGHT" AND "WAVY"-HAIRED INDIVIDUALS

by

O. W. TIEGS.

With a Foreword by Sir W. Baldwin Spencer.

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The Arunta tribe is divided into two exogamous intermarrying moieties, each of which again, in the northern part of the tribe, is divided into two classes. Those of the one moiety are called Panunga and Bultara; those of the other Purula and Kumara. In some tribes there are distinct names for the moieties, such as Dilbi and Kupathin in the Kabi tribe, Queensland, Mukwara and Kilpara in the Barkinji tribe, New South Wales. Of these the first two are said to signify respectively light and dark blood, the second two either straight and curly hair, or by some authors, eagle hawk and crow.[1] The Euahlayi tribe in New South Wales is divided into Gwaigulleah (light-blooded) and Gwaimudthen (dark-blooded) men and women.[2] In some tribes of South-west Australia the class system is said to be based on colours—white cockatoo and crow, light and dark purple.

The Arunta have no names for their moieties, but the Panunga-Bultara people are called *Gola aradjina* (straight-haired) and *Mberka oknirra* (big-bodied); the Purula-Kumara, on the other hand, are known as *Gola luda-luda* (wavy or curly-haired) and *Mberka tungwa* (small-bodied). These are not used as moiety names, but at the same time they indicate clearly the native belief that the tribe is composed of two groups, distinguished from one another by marked physical characters. It must be pointed out that the term *luda-luda* does not refer to a curly form, such as that of the hair of negroid people or the lost Tasmanians, nor does *aradjina* refer to a straight form, such as that of Malays. In fact, to the ordinary observer there is not the slightest difference to be detected between the two forms, and a native only distinguishes between them, or attempts to do so, after minute examination of individual hairs. The examination to which the native subjects the hair is not directed towards detecting a difference in the texture of the hairs, as is done, for example, by wool classers, but consists, on the contrary, in holding a hair, drawn straight out, up to the light and observing the presence or absence of waviness in its actual appearance. In a test to which I submitted the native who gave me the information, it was quite evident that he thought he could distinguish in one set of hairs a minute wavy structure, absent in the other, but, neither with the naked eye nor with the aid of a hand lens, could I distinguish any difference. Out of twenty-two that came from the *Panunga-Bultara* moiety, and, on the native theory, ought all to have been *Aradjina*, he identified seventeen as such and five as *Luda-luda;* out of seventeen that came from the *Purula-Kumara* moiety, and ought all to have been *Luda-luda*, he identified nine as such and eight as *Aradjina;* that is, out of a total number of thirty-nine, he was right in twenty-six and wrong in thirteen cases.

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[1] Rev. John Mathew, Journal R. Anthrop. Inst., vol. xl, p. 165.

[2] The Euahlayi tribe; Mrs. Langloh Parker; p. 11.

W.B.S.

When the hairs of the *Luda-luda* and *Aradjina* divisions are held up to the light and examined it is not possible to detect any difference between the two types, since, on account of the thinness of the individual hair, we cannot be certain of any change in the diameter of the hairs along their length. But when these hairs are examined under a low-powered microscope the impression is frequently gained of long waves in the hair, in the form of alternating regions of thickness and thinness. Measurement of the diameter of the hairs at various points along their length confirms this observation, and it is found that while some hairs are devoid of such waves, others show them markedly.

Hairs with or without a wave may occur upon the same individual, and of twenty hairs whose thickness was carefully measured at points 2 mm. apart along their length, five showed but little indication of a wave, and might be classed as "straight," while fourteen showed a pronounced wave, and one was intermediate between the two. Of the fourteen which showed a wave, some showed it much more markedly than did others. In fact, while some hairs are definitely "straight," and others are definitely "wavy," all intermediate gradations occur; and hairs are often found one part of which is "wavy" and another practically "straight." Of the five hairs which were "straight," three belonged to the *Aradjina* division, two to the *Luda-luda*. Of the fourteen which showed a wave, ten were *Aradjina* and four were *Luda-luda*. The intermediate one was an *Aradjina*.

The following measurements of four hairs, two from individuals belonging to each division of the tribe, as recognized by the natives, will serve to show that in both of them each type of hair occurs:

Luda-luda:

(*a*) 28, 23, 22, 27, 25½, 25, 21, 18, 22, 21½, 17, 20, 18, 18, 15, 15, 16 (i.e., wavy).

(*b*) 20, 20, 20, 20, 20, 20, 21, 21, 21, 21, 21, 21, 21, 20, 20, 20, 20, 20, 20, 20, 20, 20, 20 (i.e., straight).

Aradjina:

(*a*) 21, 20½, 20½, 20, 20, 20, 20½, 20½, 21, 20½, 20½, 20½, 20, 20, 20½, 20, 20½ (i.e., straight).

(b) 27, 21, 21, 20, 21, 20, 20½, 17, 13, 18, 20, 21, 20½ (i.e., wavy).

The numbers are micrometer measurements, and each unit corresponds to ·006μ. The waves are of irregular length, usually about a centimeter long, sometimes shorter, often much longer.

It is possible that by *Luda-luda* the native understands, not an alternating series of thick and thin regions, but a faint wave in the contour of the line of the hair in one plane, i.e., a faint indication of a zig-zag line. No indication of such a condition can be detected with the microscope.

While it may be definitely stated that wavy hairs of the type which the aboriginal thinks he observes do not occur, yet it is possible that two different types of hair may occur, in one of which there are alternating regions of darker and lighter pigmentation, giving the appearance of regions of thickness and thinness; or alternating segments of a hair may possess different optical properties, which might be similarly interpreted by the aboriginals, even if they were not visible, on account of the fineness of the hair. Such hairs might be interpreted as wavy hairs, and the aboriginal belief might be founded on fact. Such a "wave" would probably not be detectable by our ordinary methods of examining hairs, mounted on slides, in transmitted light, but should become clearly visible if a hair is held up to the light, and examined with a low-power binocular lens. I find that if this test be carried out, using a lens magnifying about three diameters, nothing that might be interpreted as waves of the kind referred to by the natives can be detected. It would appear, therefore, that the classification of individuals into "straight" and "wavy" groups is unjustified.

It is difficult to define precisely what the term *Luda-luda* means, as applied by the native to the structure of the hair, because whilst he uses this term to describe a wavy line, he also uses it for a zig-zag line. Possibly what we call wavy is not, in this case, identical with what the native calls *Luda-luda*, and certainly the latter and *Aradjina* imply to him a difference in structure that does not actually exist as distinctive of the two main divisions of the tribe. Such differences as do exist between the two types of hair are due to variations in structure so minute that they could not possibly be detected by the naked eye. Moreover, all such variations occur not only in the hair of the same individual, but in different parts of the same hair.

Concerning the belief of the aboriginals that they are composed of a wavy- and a straight-haired race, it may be pointed out that there is evidence to show that a mixture of "blood" has occurred. Thus it has recently been found by

Cleland (1) that two distinct blood-groups occur in South Australian aboriginals. This suggests that the widespread idea of the aboriginal that he is composed of two distinct races, still recognizable by their straight and wavy hair, is a tradition which has descended from a past age, when a wavy- and a straight-haired race existed, and that the two races have now largely fused. It does not seem as if much weight can be attached to the observation that some hairs are waved in the manner above described, and others, from the same individual, not (with the possible inference that this represents a fusion of straightness and waviness), since, in the first place, this kind of wave is one that the naked eye could never detect, and, secondly, similar waves can be found in the hairs of Europeans. For example, the hair of a European, measured at intervals of 2 mm. along its length, gave the following thicknesses: 18, 14, 15, 17, 17, 15, 16, 16, 16, 14, 13, 13, 12, 15, 15, 14, 15, 15, 14, 14, 15, 15, 14, 12, 13, 12, 12, 13.

The aboriginal's belief that he can recognize two different types of individuals, having respectively straight and wavy hair, cannot therefore be confirmed. But it is of interest to observe that when a hair is drawn straight out and suitably held, there occurs a play of light upon its surface. This may produce an ill-defined, dazzling effect, into which the imagination may read whatever it desires, and it probably depends upon whether the native sees waviness or straightness into the ill-defined line that he classes his individuals as *Luda-luda* or *Aradjina*.

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THE ORIGIN OF ACETALDEHYDE IN BACTERIAL AND ANIMAL METABOLISM

by

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1. *The Pyruvic Acid-Acetaldehyde Theory of Carbohydrate Breakdown.*

The pyruvic acid–acetaldehyde theory of carbohydrate breakdown, established for normal yeast fermentations by the work of Neuberg and his colleagues, has been applied also to carbohydrate breakdown by other living cells of all kinds. [See, for example Neuberg in Oppenheimer's *Handbuch der Biochemie*, 2nd ed., vol. II, and Embden (12).] In the case of bacterial and animal cells, the evidence appears to need further examination. This paper attempts such examination, and adduces some bacteriological evidence which has not previously been cited in this connection, but which seems apposite.

Acetyl methyl carbinol as an indicator of the production of acetaldehyde by bacteria.

Neuberg and his school have usually demonstrated the occurrence of acetaldehyde in fermentations and excised tissues by means of fixatives (sulphites and dimedon). A natural indicator of the presence of acetaldehyde during growth of a micro-organism is available in some cases in the substance acetyl methyl carbinol or acetoin $CH_3.CHOH.CO.CH_3$, formed by the simple condensation of two molecules of acetaldehyde. The power to make this substance, correlated by Neuberg with the presence of an enzyme called by him carboligase, is possessed by yeast (see Neuberg and Hirsch, 63), and by a number of micro-organisms, including *B. subtilis*, *B. lactis aerogenes*, *V. cholerae*, *B. diphtheriae*, and others. That acetoin originates from acetaldehyde appears now to be generally accepted. When formed it is remarkably inert. It may undergo reduction to 2.3.butylene glycol, $CH_3.CHOH.CHOH.CH_3$, or possibly oxidation to di-acetyl, $CH_3.CO.CO.CH_3$, but there is no evidence that it can be further oxidized by micro-organisms to furnish energy. Meyerhof (50) has shown that it does not increase the oxygen uptake of yeast, and the carbon-dioxide output of yeast is also unaffected by it (Neuberg and Kobel, 64). Di-acetyl or acetoin can be added to a yeast fermentation and recovered again mainly as butylene glycol (Neuberg and Nord 67, Neuberg and Kobel 64). The present writer has followed the course of production of acetoin and butylene glycol from sugar by a soil bacillus, and has found that the sum of the two reaches a maximum at the time when the last of the sugar disappears, and does not subsequently diminish.

To use acetoin as an indication that acetaldehyde has been formed in the course of a fermentation is perhaps better than adding sulphite as a fixative. The normal balance of reactions is not altered; that this may be a real danger in the presence of sulphite is suggested by the work of Bleyer and Schmidt (6) on the behaviour of sugars in sulphite-containing solutions. No large accumulation of acetaldehyde is necessary for acetoin to be formed; traces can be demonstrated in well-aerated normal yeast fermentations. When formed it is stable, and easily detected in minimal quantities by the method devised by Lemoigne (36), and improved by Kluyver, Donker, and Visser t'Hooft (34). The principle of the method is oxidation of acetoin to diacetyl, and precipitation of di-acetyl as nickel-dimethyl-glyoxime.

Examining yeast as a test case, it is found that observations on acetoin and on sulphite fixations give concordant results. Traces of acetoin may be present in normal fermentations; the quantity can be increased by aeration or by adding hydrogen acceptors, such as methylene blue, sulphur, or more acetaldehyde; less acetaldehyde is then reduced to ethyl alcohol, and more remains to be condensed into acetoin. In alkaline fermentations, parallel with a small accumulation of acetaldehyde, runs a formation of acetoin. The action of yeast on pyruvate leads to the production of acetoin in large quantities. (See papers by Neuberg and his collaborators, in the Biochemische Zeitschrift, from 1921 onwards.)

When the acetoin-producing bacteria are investigated with regard to acetaldehyde production, it is found that acetoin (and one may conclude acetaldehyde) is formed freely from sugars and glycerol, but only sparingly from lactate and pyruvate. The experiments of Harden and Norris (27) show that *B. lactis aerogenes* forms acetoin or butylene glycol from a variety of sugars, from glycerol, and from acetaldehyde. Ethelyene glycol also gives rise to butylene glycol, the preliminary reaction being, one may suppose, the removal of the elements of water to leave acetaldehyde:

$$CH_2OH.CH_2OH \rightarrow CH_3.CHO + HOH.$$

No acetoin was formed from citrate or malate. A peptone medium was used throughout. Lemoigne (35) found acetoin after growth of *B. subtilis* on peptone-sucrose or peptone-glycerol media, but it was not produced in a peptone-lactate medium. Later (37) he succeeded in obtaining minute quantities of acetoin after long continued growth of an organism of the *subtilis* group on a calcium lactate-peptone medium. Observations by the present writer on the acetoin-forming soil bacillus previously mentioned show that it does not make acetoin from lactate or pyruvate, in spite of excellent growth, while from a 2% glucose medium up to 10% of the sugar may be recovered as acetoin and butylene glycol. Acetoin is also formed from glycerol, but not from succinate. In view of the increased yield of acetoin obtained by aeration of yeast fermentations, the soil bacillus was cultivated on lactate and on pyruvate in a stream of oxygen, but still not a trace of acetoin could be detected by the very sensitive method of Lemoigne. It was, however, found possible to identify minute amounts of acetoin in a pyruvate medium after growth of *B. subtilis*, an observation which may be associated with Lemoigne's on calcium lactate.

In the case of *B. subtilis*, it is perhaps permissible to speculate on lines suggested by Meyerhof's work: may not the acetoin and its parent acetaldehyde arise from sugar formed by synthesis from the lactate or pyruvate, rather than directly from these substances? Organisms of a strongly aerobic and non-fermenting type, such as *B. subtilis*, may owe these characteristics partly to enhanced synthetic powers; it may be that intermediate products do not accumulate in the medium because they are rapidly resynthesized to carbohydrate as fast as they are formed, a part undergoing oxidation to provide energy for the synthesis, as with muscle. Meyerhof has shown that lactic and pyruvic acids are readily synthesized to carbohydrate both by yeast and by muscle. The distinction between non-fermenting aerobic bacteria (e.g. *B. subtilis* and the Timothy Grass bacillus), and fermenting facultative anaerobes (e.g. *B. coli* and the acetoin-forming soil bacillus) may be in part the same as the distinction between the wild oxidizing yeasts and the cultivated fermenting yeasts (see Meyerhof, 50).

Other Observations on the Production of Acetaldehyde by bacteria.

In view of the results obtained with acetoin, it seemed worth reconsidering experiments made with bacteria in presence of sulphite as a fixative for acetaldehyde. When these were surveyed it was noticeable that, as with acetoin, positive results were always obtained when acetaldehyde was sought in media containing sugar or glycerol, but that with lactate and pyruvate the results were doubtful or negative. Thus Neuberg and Nord (66) isolated acetaldehyde by means of sulphite from fermentations of sugars, mannitol, and glycerol, by *B. coli*, and the bacilli of dysentery and gas gangrene. Peterson and Fred (71) grew three pentose-fermenting bacteria (*B. acetoethylicum*, *Lactobacillus pentoaceticus*, and an organism of the *colon-aerogenes* group) on media containing pentose or hexose, and obtained acetaldehyde by the use of sulphite; they failed, however, to demonstrate any acetaldehyde in pyruvate media, in spite of good growth. Nagai (53) was able to isolate considerable amounts of acetaldehyde after growth of *B. coli* and *B. lactis aerogenes* on sugar-peptone media in presence of sulphite, and smaller amounts after growth on lactate media. Like so much other work, Nagai's is made difficult of interpretation by casual addition or absence of peptone.

Both acetoin and sulphite experiments seem to show that acetaldehyde is more freely formed by bacteria from sugars and glycerol than from lactic and pyruvic acids. With the latter may be associated substances such as succinic, malic, and fumaric acids, which from the work of Quastel (72, 73) would seem to be normally metabolized by bacteria *via* oxalacetic and pyruvic acids. That lactic acid belongs to the same group, and is also normally oxidized by bacteria and yeast to pyruvic acid, is clear from the observations of Mazé (48, 49), Quastel, Stephenson and Whetham (75), and others.

The Action of Plants on Pyruvic Acid.

The contrast between the action of bacteria on pyruvic acid and the effect of a yeast fermentation of pyruvic acid is very marked. An experiment described by Neuberg and May (1923) allowed the following balance to be established in such a fermentation. From 13·2 gr. of pyruvic acid were obtained:

4·82 gr. of carbon-dioxide.
2·86 gr. of acetaldehyde (as the dimedon compound).
2·07 gr. of acetoin.
3·36 gr. of unchanged pyruvic acid.

13·11 gr.

Other α-keto acids are attacked in the same way by yeast The aldehyde resulting from decarboxylation is usually partly recovered as such, and partly as the corresponding alcohol and acid after a Cannizarro reaction between two molecules of aldehyde (compare Neuberg's "third form of fermentation"). Some of the aldehyde may undergo condensation. α-Keto-butyric, α-keto-valeric, α-keto-caproic, and aromatic α-keto acids were used by Neuberg to demonstrate the decarboxylating action of yeast.

The experiments of Nagayama (54) show that moulds such as *Mucor* and *Aspergillus* exhibit towards pyruvic acid a grade of decarboxylative activity similar to that of yeast. He grew six species of moulds on pyruvate media, and isolated 4–24% of the theoretical yield of acetaldehyde; this amount was somewhat increased in presence of sulphite. The resemblance of moulds to yeast is further emphasized by the work of Neuberg and Cohen (59), who cultivated a number of moulds on sugar-containing media in presence of sulphite, and obtained large amounts of acetaldehyde in every case. Many species behaved as yeast does in presence of sulphite, changing sugar to equimolecular quantities of glycerol and acetaldehyde (Neuberg's "second form of fermentation"). Other species with a more aerobic type of metabolism produced no glycerol.

A definite anaerobic production of acetaldehyde and carbon-dioxide from both pyruvate and glucose is also reported for the green plant. For example, Zaleski and Marx (96, 97) showed that powdered peas and beans produced acetaldehyde and carbon-dioxide anaerobically from pyruvate solutions. The same was reported by Bodnár (7) for enzyme preparations of potatoes and sugar beet. Neuberg and Gottschalk (62) made a sterile powder of peas, and suspended it in a glucose solution under anaerobic conditions. After three days they were able to isolate from one experiment 5·5 mg. of acetaldehyde and 3·0 grm. of alcohol, while in a parallel experiment, in presence of sulphite, they found 164·2 mg. of acetaldehyde and only 0·33 grm. of alcohol.

In the plant kingdom, then, the power to decarboxylate pyruvic acid occurs from yeasts and moulds upwards. Acetaldehyde may be produced anaerobically from either pyruvate or glucose, and is a stage in the production of alcohol which is characteristic of the plant under anaerobic conditions. That acetaldehyde is formed also during the normal (aerobic) respiration of the green plant is shown by Klein (33). Yet even with yeast, it is doubtful whether pyruvic acid should be considered an invariable stage between carbohydrate and acetaldehyde. In normal fermentations by yeast juice the intervention of pyruvic acid seems proved by the experiments of v. Grab (24), who used β-naphthylamine as a means of fixation, and obtained about 4% of the sugar fermented as a condensation product of pyruvic acid (α-methyl, β-naptho-cinchoninic acid). On the other hand, Hägglund and Augustsson (26) find that at P_H above 7 yeast ferments pyruvic acid very slowly. This they suggest might be explained on the basis of observations by Henri and Fromageot (28) as due to the preponderance of the enol isomeride of pyruvic acid at high P_H, as compared with the preponderance of the apparently more easily decarboxylated keto isomeride at low P_H. If pyruvic acid were formed by yeast during alkaline fermentation, one might thus expect that it would accumulate. Kato (32) and others have found that the accumulation of pyruvic acid in bacterial fermentations is favoured by neutral or alkaline conditions. One is inclined to wonder whether the decarboxylative activity of the plant towards pyruvic acid is not partly due to the more acid reaction of the plant cell, and the consequent shift of equilibrium towards the keto form of pyruvic acid.

The Relations of Acetaldehyde and Pyruvic Acid in the Animal Cell.

With bacteria it is clear that the production of acetaldehyde from pyruvic acid does not occur to anything like the same extent as in the plant. In the animal, also, the power of decarboxylate α-keto acids is never prominent, though the occurrence of acetaldehyde during oxidation of carbohydrate is well established. For example, Hirsch (29) isolated acetaldehyde from chopped frog muscle, using dimedon as a fixative. Neuberg and Gottschalk (60) obtained acetaldehyde from liver pulp incubated with sulphite, and found an increased yield in presence of glycogen and other substances. The production of acetaldehyde was inhibited by cyanide (Gottschalk, 23). In a later paper, Neuberg and Gottschalk (61) claim that at least 40% to 50% of the carbohydrate oxidized by chopped frog muscle passes through the stage of acetaldehyde. This amount was actually isolated by the use of calcium sulphite, which is by no means a completely efficient fixative. Stepp and Feulgen (88) found small quantities of acetaldehyde in urine. Fabre (16) and Supniewski (91) report acetaldehyde in blood; Gee and Chaikoff (22) confirm its presence in amounts of 2 to 5 mg. per litre.

But turning to pyruvic acid, the experiments of Gottschalk (23) show that liver tissue is capable of changing it into acetaldehyde and carbon-dioxide in very small amounts only. He incubated liver pulp with sulphite, and found that acetaldehyde could be isolated under aerobic conditions in amounts of about 2 mg. per 100 grm. of tissue; in presence of pyruvate (equivalent to 0·2 gr. of pyruvic acid), acetaldehyde was formed anaerobically (2 mg.), and the aerobic yield was increased to 4 mg. Wieland (94), working with a variety of catalysts, found that neither palladium black, charcoal, nor tissues, with the sole exception of unwashed liver tissue, could catalyze the anaerobic decomposition of pyruvic acid into acetaldehyde and carbon-dioxide. On the other hand, he found that all his catalysts, including boiled tissues, could rapidly split off carbon-dioxide from oxalacetic acid:

$$COOH.CH_2.CO\ COOH \longrightarrow CH_3.CO.COOH + CO_2.$$

This point is of interest, since the breakdown of oxalacetic acid is often classed with that of pyruvic acid as indicating the presence of "carboxylase." It would be well to adopt the division into α and β carboxylases made by some writers, and to recognize that oxalacetic acid may be decomposed to pyruvic acid and carbon-dioxide or acetoacetic acid to acetone and carbon-dioxide, by tissues which cannot catalyze the decarboxylative breakdown of pyruvic acid. In other words, carbon-dioxide is more easily split off from β than from α-ketonic acids, a fact which is obvious from the behaviour of β-ketonic acids *in vitro,* and which could no doubt find explanation on the lines of the electronic considerations developed by Quastel (73).

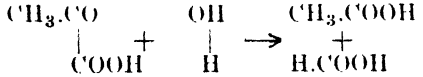
A case illustrating the need for definition is the work of Mayer (46) on the anaerobic breakdown of oxalacetic acid by liver and muscle tissue. He found that when chopped tissue was incubated for 24 hours with oxalacetic acid in buffered solution, about 1 molecule of carbon-dioxide was produced per molecule of oxalacetic acid, as compared with half this amount when oxalacetic acid was incubated alone. The experiments are taken to prove the presence of "carboxylase," but seem rather to suggest that true α-carboxylase is not markedly active under these conditions. A slight breakdown of pyruvic acid probably did occur, since in some experiments rather more than 1 molecule of carbon-dioxide was produced from 1 molecule of oxalacetic acid, and also since a small amount of oxalacetic acid is not broken down, but is converted into malic acid in presence of tissues (Mayer, 47).

Absence of carboxylase from a liver carcinoma is reported by v. Euler and Myrbäck (15); no measurable amount of carbon-dioxide was developed from a pyruvate solution within 24 hours. These authors are of the opinion that the carboxylase found in animal tissues acts on some other substrate than pyruvic acid, and that the acetaldehyde formation observed by Neuberg and Gottschalk in animals is not connected with carboxylase. Shaffer also, after reviewing the evidence, write (81): "There are difficulties in the acceptance of the view that glucose is oxidized via . . . pyruvic acid and acetaldehyde." Gottschalk, writing in Oppenheimer's *Handbuch der Biochemie* (2nd ed., vol. II) on the breakdown of carbohydrate by animal cells, concludes that facts do not yet permit of a decision as to whether pyruvic acid is the precursor of acetaldehyde in the oxidative breakdown of carbohydrate by muscle.

The Breakdown of Pyruvic Acid by Bacteria.

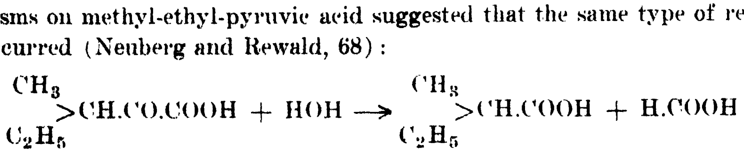
Before considering an alternative source for acetaldehyde in bacterial and animal metabolism, some evidence as to the non-decarboxylative breakdown of pyruvic acid may be discussed.

Neuberg in 1914 (56) showed that pyruvic acid was broken down to acetic and formic acids by a mixed culture of putrefactive organisms. He explained the products as arising by a hydrolysis of the following type:



$$\begin{array}{c}CH_3.CO\\ |\\ COOH\end{array} + \begin{array}{c}OH\\ |\\ H\end{array} \longrightarrow \begin{array}{c}CH_3.COOH\\ +\\ H.COOH\end{array}$$

A similar reaction was found to occur with other α-keto acids: for example, 20% of the theoretical yield of propionic acid was isolated from a fermentation of α-keto-butyric acid, along with some formic acid, carbon-dioxide, and hydrogen (Neuberg, 57). Again, the products of the action of mixed putrefactive organisms on methyl-ethyl-pyruvic acid suggested that the same type of reaction had occurred (Neuberg and Rewald, 68):

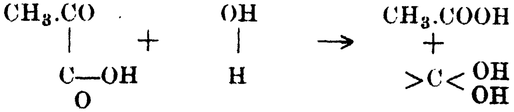


$$\begin{array}{l}CH_3\\ \\ C_2H_5\end{array}\!\!>CH.CO.COOH + HOH \longrightarrow \begin{array}{l}CH_3\\ \\ C_2H_5\end{array}\!\!>CH.COOH + H.COOH$$

It seems worth while to inquire if such a change to the next lower fatty acid + formic acid is not a more usual path for the breakdown of α-keto acids by the animal and bacterial cell than decarboxylation to an aldehyde + carbon-dioxide. It must be recognized that decarboxylation may be followed by oxidation of the aldehyde to acid (or a Cannizarro reaction of 2 molecules of aldehyde to give alcohol and acid); the products after such a reaction, namely, acid and carbon-dioxide, resemble those of the suggested hydrolysis after oxidation of formic acid has occurred. On the other hand, it is equally possible to suppose that any acetaldehyde produced from pyruvic acid is derived from acetic acid by reduction, though, as we have already seen, acetaldehyde is seldom reported as arising from pyruvic acid.

Thus it is obviously difficult to draw hard and fast lines of distinction. Nevertheless, some evidence is available. Besides Neuberg's work with mixed cultures of bacteria, there are other examples of hydrogen being set free from fermentations of pyruvic acid, and though hydrogen may be readily accounted for as arising from formic acid, it is difficult to see how its origin could be explained on the theory of decarboxylation. Karczag and Móczár (31), using peptone media, found that gas was produced from pyruvate by the same organisms which produce gas from sugar, e.g. *B. coli, B. paratyphoid B.*, and *B. enteriditis* (Gaertner) (not by *B. typhosus, B. dysenteriae, Streptococci*, or *Staphylococci*). The gas was found to contain a large proportion of hydrogen. These authors conclude that "die Brenztraubensäure durch Bakterien eine Veränderung erlitt, die von der Veränderung die diese Substanz durch Hefe erleidet, biologisch und chemisch zu unterscheiden ist." Peterson and Fred (71) found hydrogen produced from pyruvate by an organism of the *colon-aerogenes* group. The present writer has observed the production of hydrogen in considerable quantity from a pyruvate-inorganic medium by the soil bacillus already mentioned. Traces of formic acid can be identified in addition.

On the other hand, many species of bacteria produce no gas when grown on pyruvate-inorganic medium with Durham tubes, including some species which Pakes and Jollyman (70) describe as capable of breaking down formic acid to give hydrogen. And again, there is no accumulation of formic acid in pyruvate media after growth of organisms, which according to Pakes and Jollyman are unable to attack formate. It should be noted, however, that Pakes and Jollyman seem to have employed the production of hydrogen as the only criterion of the breakdown of formate. It is conceivable that some organisms reported by them as not attacking formate did so in reality, transferring hydrogen to acceptors in the medium; of these there was no lack, as 2% peptone was used. Similarly, we may imagine that formic acid produced from pyruvate might be oxidized as soon as it is formed, whether by hydrogen acceptors or by oxygen. Emulsions of *B. coli* and *B. pyocyaneus* will very readily transfer hydrogen from formic acid to methylene blue (see Quastel and Whetham, 77, 78). The concentration of formate in Pakes and Jollyman's experiments is not definitely stated, but seems to have been from 0·5% to 2·0%. It is likely that organisms might give off hydrogen from formate present in such large amounts, but might be able to oxidize it as fast as it was produced from pyruvate. Especially does this seem likely, since formic acid is most easily pictured as arising from pyruvic in "activated" or "nascent" form:



$$\begin{array}{c} CH_3.CO \\ | \\ \underset{O}{C}\text{—}OH \end{array} + \begin{array}{c} OH \\ | \\ H \end{array} \rightarrow \begin{array}{c} CH_3.COOH \\ + \\ >C<\begin{smallmatrix} OH \\ OH \end{smallmatrix} \end{array}$$

There is a further reason for desiring more information on the subject of formic acid breakdown by bacteria than is available in the work of Pakes and Jollyman: their experiments appear to have been performed under anaerobic conditions, hence the true aerobes are all classed as not attacking formate. Unpublished experiments of Stephenson and Whetham show that at least one aerobe (the Timothy Grass bacillus) can oxidize low concentrations of formic acid when glucose is also present to provide a suitable source of carbon for growth. The whole subject of the breakdown of formic acid by bacteria has remained almost untouched since the time of Pakes and Jollyman, and would repay fresh investigation.

The presence of acetic acid in fermentations of pyruvic acid has often been reported, but, as noted above, this may be explained on the basis of either theory. Acetic and formic acids are among the products of breakdown of succinic acid (E. C. Grey, 25), and fumaric acid (Quastel, 72), both of which substances are probably metabolized by bacteria *via* pyruvic acid (see above).

Observations, probably relevant but not easy to interpret, are those on changes of hydrogen ion concentration in pyruvate media during the growth of bacteria of various species. Some show an immediate increase of alkalinity from an original P_H of 7·4 (*B. pyocyaneus, B. fluorescens*); some grow for a day or two without any change of P_H, and then produce alkalinity (*B. subtilis*); others cause a fall in P_H, which may extend below P_H 6·5, and last for a week or more (*B. coli, B. prodigiosus*, the acetoin-forming soil bacillus). In all cases the final reaction is alkaline, marking the accumulation of base when the pyruvic acid is fully oxidized. The formation of two acids from one by hydrolysis would account for the fall of P_H; it is, however, difficult to demonstrate more than traces of formic acid. The acidity may perhaps rather be attributed to simultaneous removal of organic acid for synthetic processes, accumulation of acetic acid, and utilization of ammonia; where the medium becomes alkaline, one may picture a more rapid removal of acetic acid by oxidation or synthesis. Such points are susceptible of experimental proof. A low P_H during metabolism of pyruvic acid by *B. coli* and other species was recorded by Karczag (30, 31). It is of interest that the P_H changes produced by various bacteria during growth on a pyruvate-ammonia medium are similar to those produced by each species in Cole and Onslow's tryptic broth (casein digest). One may perhaps explain this by recourse to the work of Neubauer (55), and may regard an amino acid mixture as a potential mixture of ammonium salts of α-keto acids.

The energy relationships of the products of pyruvic acid breakdown are worth consideration, especially in view of the fact that anaerobic growth of facultative anaerobes is possible where pyruvic acid is the sole source of carbon (Aubel and Salabartan, 2) (Quastel and Stephenson, 74). The heat of combustion of pyruvic acid, recently determined by Blaschko (5), is identical with that of acetaldehyde; decarboxylation consequently occurs without energy transfer. On the other hand, both the formation of alcohol and acetic acid from acetaldehyde, and the formation of acetic and formic acids from pyruvic acid, are processes involving output of energy:

$$2\ CH_3.CHO + HOH \rightarrow C_2H_5.OH + CH_3.COOH + 22\ Cal.$$

$$CH_3.CO.\ COOH + HOH \rightarrow CH_3.COOH + H.COOH + 6{\cdot}5\ Cal.$$

Either decarboxylation or hydrolytic breakdown could therefore provide energy for synthetic processes during anaerobic growth on pyruvic acid. A third possibility is that suggested by Quastel and Stephenson (74):

$$2\ CH_3.CO.COOH + HOH \rightarrow CH_3.CHOH.COOH + CH_3COOH + CO_2 + 15\ Cal.$$

Investigation of the products left after anaerobic growth on pyruvic acid might throw light on the processes of breakdown.

The Breakdown of Pyruvic Acid by the Animal Cell.

Passing now to the metabolism of pyruvic acid by the higher animal, it is first to be noted that pyruvic stands in close relationship to lactic acid, and usually undergoes synthetic rather than destructive treatment. Thus in the diabetic or phloridzinised organism, pyruvate gives rise to almost as large an excretion of "extra glucose" as lactic acid (Dakin and Janney, 10). Mayer (45) found lactic acid in the urine of rabbits after administering pyruvic acid, and Embden and Oppenheimer (14) showed an increase of *d*-lactic acid after perfusing surviving dog's liver with blood containing pyruvate. Meyerhof's recent work (Meyerhof, Lohmann and Meier, 51) places pyruvic acid with lactic acid as the only substances which can be re-synthesized to glycogen by muscle.

It is possible that the breakdown of pyruvic acid may be effected by the animal to a small extent under certain conditions. Experiments such as those of Tschernorutsky (92) with liver, and Maeda (44) with placenta, merely show disappearance of pyruvate and production of carbon-dioxide. Embden and Oppenheimer (13) perfused surviving livers with pyruvate, and found an increased production of acetoacetic acid, which they attributed to formation of acetaldehyde, condensation to aldol, and subsequent oxidation:

$$2\ CH_3.CO.COOH \rightarrow 2\ CH_3.CHO + 2\ CO_2$$

$$2\ CH_3.CHO \rightarrow CH_3.CHOH.CH_2.CHO$$

$$CH_3.CHOH.CH_2.CHO + O_2 \rightarrow CH_3.CO.CH_2.COOH + HOH$$

In view of the results of Loeb (39), however, the production of acetoacetic acid from pyruvate might also be explained by direct condensation of acetic acid arising from pyruvate:

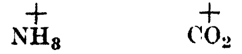
$$2\ CH_3.CO.COOH + 2\ HOH \rightarrow 2\ CH_3.COOH + 2\ H.COOH$$
$$2\ CH_3.COOH \rightarrow CH_3.CO.CH_2.COOH + HOH$$

Loeb (39) obtained increased acetoacetic acid on adding acetate to the blood perfusing a surviving liver; 3% to 10% of the acetic acid went to acetoacetic, and 30% to 90% disappeared. The formation of acetoacetic acid from acetaldehyde by the surviving liver, observed by Friedmann (21), appears to be less regular and less extensive than the formation from acetic acid. One might even suppose that acetoacetic acid does not come from acetaldehyde *via* aldol, but *via* acetic acid derived from acetaldehyde by a Cannizarro reaction. That liver tissue can catalyze the production of alcohol and acetic acid from acetaldehyde is well established.

In the earlier perfusion experiments no attempt was made to estimate disappearance of pyruvic acid. Simon and Aubel (82) found that only 40% of pyruvate injected into a dog or rabbit could be recovered after 1 hour, but they did not investigate its fate; synthesis rather than breakdown may have been involved. Levene and Meyer (38), using aseptic precautions, incubated kidney pulp and leucocytes with sodium pyruvate; there was no production of carbon-dioxide, and no diminution in pyruvic acid as estimated by the total amount of permanganate required to oxidize substances left after precipitation of protein. These results were confirmed for liver and muscle tissue by Simon and Aubel (83), using the hydrazone of pyruvic acid as a means of estimation.

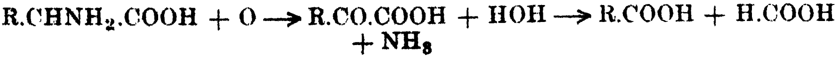
With the higher animal, as with bacteria, the behaviour of succinic acid indicates that it may be metabolized *via* pyruvic acid. In the diabetic, succinic acid gives rise to glucose; when perfused through a surviving liver, small amounts of acetoacetic acid are formed from it (Ohta, 69). Malonic acid also gives acetoacetic acid in perfusion experiments, no doubt by virtue of a breakdown to acetic acid and carbon-dioxide (Momose, 52).

The general question of the intermediate metabolism of α-keto acids in the body is partly treated by Neubauer (55) in his work on amino acids. His evidence shows that amino acids probably undergo oxidative deamination to the corresponding α-keto acid; also that under certain conditions amino acids give rise to the fatty acid with one carbon atom less. He supposes that the α-keto acids arising from amino acids go by decarboxylation and subsequent oxidation to the fatty acid next lower:



$$R.CHNH_2.COOH + O \rightarrow \underset{NH_3}{\underset{+}{R.CO.COOH}} \rightarrow \underset{CO_2}{\underset{+}{R.CHO}} + O \rightarrow R.COOH$$

One may offer the following alternative scheme for the final stages:



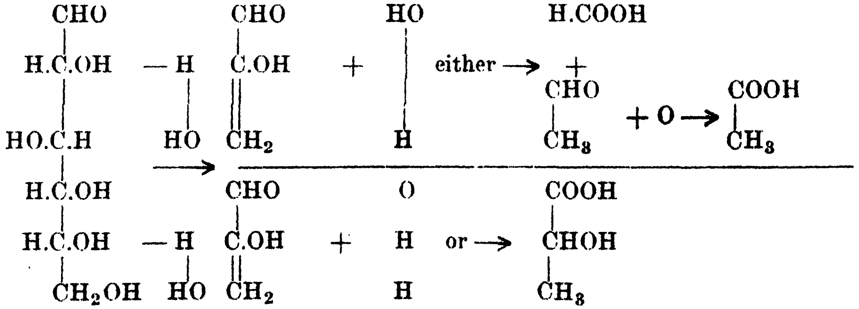
$$R.CHNH_2.COOH + O \rightarrow \underset{+\ NH_3}{R.CO.COOH} + HOH \rightarrow R.COOH + H.COOH$$

Formic acid will receive further attention in the next section, but one point may be raised here. On the basis of Wishart's work, Oppenheimer writes in *Die Fermente* (5th ed., p. 1726) that formic acid is inactive as a hydrogen donator to methylene blue in presence of animal tissues, in contrast to the extreme activity found for formates by Quastel and Whetham (77, 78) in presence of emulsions of *B. coli* or *B. pyocyaneus*. Reference to Wishart's paper (95) shows that although formic acid was inactive in presence of muscle extracts, yet in presence of liver extracts it was a better hydrogen donator than any other substance except succinate. Batelli and Stern (4) have shown that animal tissues possess to a marked extent the power of oxidizing formic acid in presence of hydrogen-peroxide. Formic acid administered to the intact animal in moderate doses is readily oxidized. If, then, one postulates a split to fatty acid + formic acid as the first stage in the breakdown of α-keto acids produced in the liver by deamination of amino acids, there is no difficulty in picturing the immediate destruction of such formic acid.

On the whole, one is tempted to the view that the breakdown of α-keto acids by the animal body and the bacterial cell may follow the hydrolytic path indicated by the researches of Neuberg on bacteria, rather than the decarboxylative path indicated by his discoveries with yeast. If pyruvic acid be broken down to acetic and formic acids, what is to replace it as precursor of the acetaldehyde found during metabolism of carbohydrate?

2. *The Origin and Fate of Acetaldehyde.*

Graham Lusk, in his paper, "Die Energiequelle bei der Muskelarbeit" (1925), formulates the following scheme for carbohydrate breakdown by muscle:



Methyl-glyoxal formed from glucose may be changed (*a*) under asphyxial conditions to lactic acid, or (*b*) under aerobic conditions to acetaldehyde and formic acid. Lactic acid is not oxidized in the body, and is only removed by re-synthesis to carbohydrate. Acetaldehyde is formed during the oxidation of methy-glyoxal, and undergoes further oxidation to acetic acid. It is not produced under asphyxial conditions; if it were, the body would make alcohol from it, and alcohol is only found in small quantities even after death from asphyxia.

Of direct evidence for this scheme there is none. The question of the oxidation of methyl-glyoxal has been neglected in favour of investigations on the action of glyoxalase in changing methyl-glyoxal to lactic acid. A single fact about phenyl-glyoxal is perhaps pertinent. Dakin and Dudley (9) administered doses of 1·0 to 1·5 gr. of phenyl-glyoxal to rabbits, and besides about 0·5 gr. of mandelic acid, they were able to recover from the urine about 0·75 gr. of hippuric acid, corresponding to 0·5 gr. of benzoic acid. Mandelic and benzoic acids in this experiment may correspond to lactic and acetic acids in the scheme given above.

But a certain amount of indirect evidence is available, and on the whole the scheme appears to accord well with many facts besides those assembled by Lusk. It provides at least a useful alternative hypothesis in place of the pyruvic acid-acetaldehyde theory. As well as explaining the presence of acetaldehyde where carbohydrate is being oxidized, Lusk's scheme also accounts for the equally widespread occurrence of formic acid.

Production and Breakdown of Formic Acid.

This substance has been reported from bacterial fermentations of all kinds. It may be formed (and can be broken down) by yeast growing in aerobic conditions (see Franzen and Steppuhn, 20). Steppuhn and Schelbach (90) found formic acid in the urine of normal dogs and rabbits and in autolyzing tissue; they observed an increased excretion of formic acid by a dog after addition of glucose to the diet, and they claim that formic acid is an intermediate product of carbohydrate breakdown. Dakin, Janney and Wakeman (11) found 80 to 118 mg. of formic acid per day in normal human urine. They give results showing that the daily excretion of formic acid by a dog dropped in starvation to a constant level of 9 mg.; this was increased to 27 mg. on a meat diet and 51 mg. on a potato diet. Formic acid excretion also rose after intravenous injections of glucose. Stepp (87) discovered small amounts of formic acid in normal human blood. It has been shown in the previous section how formic acid might arise in protein metabolism, and Lusk's scheme suggests an origin for it in carbodydrate metabolism; it would appear that small amounts may in each case escape oxidation. Since formic acid in small doses is completely oxidized by the animal body, one may perhaps conclude that the normal excretion represents the breakdown of considerable amounts.

All workers on the subject have recognized that acetaldehyde is, in the animal body, an oxidation product of glucose. On the pyruvic acid theory, oxidation must occur in order that pyruvic acid should be formed from glucose. On Lusk's scheme, it is not at first sight clear why oxidation should be necessary to produce acetaldehyde, but consideration of the thermal relations illuminates the situation. The reaction glucose → 2 acetaldehyde + 2 formic acid is endothermic: $C_6H_{12}O_6 \longrightarrow 2\ CH_3.CHO + 2\ H.COOH - 6{\cdot}8$ Cal. It can thus only proceed if energy is simultaneously obtained from some other reaction. Neglecting acetaldehyde for a moment, one may consider the various possible modes of breakdown of formic acid.

1. With bacteria which liberate hydrogen from formic acid, it would seem that energy for production of acetaldehyde must be obtained from some source other than formic acid, e.g., from the split of carbohydrate to lactic acid. Some bacteria which set free hydrogen during fermentation of sugar, e.g., *B. coli*, change about half the sugar fermented to lactic acid. Aubel (1) interprets the breakdown of sugar by *B. coli* as involving an exothermic split to lactic acid, coupled with an endothermic formation of pyruvic acid and hydrogen; aerobically the hydrogen is oxidized, anaerobically it is set free as such. One may interpret his experimental results equally well on the basis of an exothermic split to lacid acid, coupled with an endothermic production of acetaldehyde and formic acid. The pyruvic acid, he finds (using a medium containing chalk), may arise partly, as he suggests, by oxidation of methyl-glyoxal; another possibility, especially in the aerobic experiments, is oxidation of lactic acid, to which may be due the lower yield of lactic acid aerobically.

2. With other bacteria, and in part with *B. coli*, etc., formic acid is destroyed anaerobically, but hydrogen is not set free. This must involve an oxidation-reduction, from which it may be possible to obtain energy, e.g.:

$$CH_3.CHO + H.COOH \longrightarrow C_2H_5.OH + CO_2 + 15\ \text{Cal.}$$
$$CH_3.CHOH.CH_2.COOH + H.COOH \longrightarrow CH_3.CH_2.CH_2.COOH + HOH + CO_2 + 27\ \text{Cal.}$$
$$COOH.CH = CH.COOH + H.COOH \longrightarrow COOH.CH_2.CH_2.COOH + CO_2 + 27\ \text{Cal.; etc., etc.}$$

Comparing the first equation with that previously given for the breakdown of glucose to acetaldehyde and formic acid, it is apparent that the reduction of acetaldehyde to ethyl alcohol by formic acid yields sufficient energy to provide for a further production of acetaldehyde from glucose—as is, of course, to be expected, since the initial and final stages are those of alcoholic fermentation. It may be that a scheme of this kind actually represents the course of events in alkaline fermentation. It has been suggested above that pyruvic acid may not be an intermediate product in Neuberg's "second" and "third forms of fermentation," and one might assume in these cases a masked formation of formic acid leading to immediate reduction of a ½ sugar molecule to glycerol:

$$C_6H_{12}O_6 \longrightarrow 2\ C_3H_6O_3$$
$$C_3H_6O_3 \longrightarrow CH_3.CHO + H.COOH$$
$$C_3H_6O_3 + H.COOH \longrightarrow C_3H_8O_3 + CO_2$$

3. Finally, formic acid may be oxidized to carbon-dioxide and water, giving ample energy to further the split of glucose to acetaldehyde and formic acid:

$$H.COOH + O \longrightarrow HOH + CO_2 + 63 \text{ Cal.}$$

One must assume this to be somehow the ultimate mode of breakdown in the animal body. Muscle seems unable to catalyze the transfer of hydrogen from formic acid to methylene blue. In the liver, the work of Wishart indicates that some transfer of hydrogen from formic acid to hydrogen acceptors may occur, and it is actually found that production of alcohol under anaerobic conditions—always small in animal tissues—is largest in the liver. It appears as though acetaldehyde were not produced elsewhere except by a reaction coupled with direct oxidation of formic acid.

Production of Acetic Acid.

It is indeed one of the essentials of Lusk's theory that acetaldehyde should only be produced under aerobic conditions, and should undergo immediate oxidation to acetic acid. Acetaldehyde is toxic to the animal body in fairly low concentration, and if large doses are given to an animal there is much disturbance of metabolism; on the other hand, large doses of acetate are easily tolerated and rapidly oxidized. See, e.g. Lusk (42) and Loeb (39) quoted above. The further fate of acetic acid becomes a matter of great interest. Some evidence of its employment in synthetic operations may be discussed, with a view to emphasizing the importance of acetic acid rather than acetaldehyde in such processes.

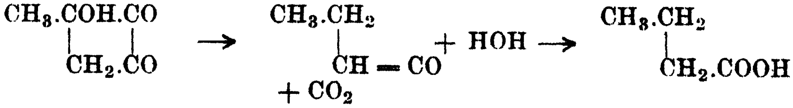
Acetic Acid in Synthetic Fermentations.

Bakonyi (3), working on the acetone-ethyl alcohol fermentation of starch by *B. macerans* and *B. acetoethylicum*, found that in presence of chalk the proportion of the products was 2 molecules of alcohol to 1 molecule of acetone. Acetaldehyde and acetaldol, $CH_3.CHOH.CH_2.CHO$, were added to fermentations and completely recovered as ⅓ acetone, ⅔ ethyl-alcohol. Added calcium acetate was completely recovered as acetone. Bakonyi supposes that acetaldehyde is produced according to the scheme of Neuberg, hydrogen being set free; 2 molecules of acetaldehyde then undergo condensation to aldol, which yields acetic acid and ethyl-alcohol by hydrolysis; 2 molecules of acetic acid (1 of calcium acetate) give 1 molecule of acetone:

$$2\ CH_3.CHO \longrightarrow CH_3.CHOH.CH_2.CHO$$
$$CH_3.CHOH.CH_2.CHO + HOH \longrightarrow CH_3.CH_2.OH + CH_3.COOH$$
$$(CH_3.COO)\ 2.Ca \longrightarrow CH_3.CO.CH_3 + CaCO_3$$

An alternative and simpler hypothesis is to suppose that acetaldehyde undergoes a Cannizarro reaction to give alcohol and acetic acid, without the intervention of aldol. Be that as it may, the fact remains that added acetic acid is quantitatively changed to acetone. Acetoacetic acid is an obvious intermediate stage. Here, as with liver perfusions (see above), acetic acid rather than acetaldehyde is indicated as the starting point for synthesis of acetoacetic acid. It may be noted also that acetone is produced from pyruvate by *B. acetoethylicum* (Speakman, 85), yet no acetaldehyde is found when this organism is grown on pyruvate in presence of sulphite (Peterson and Fred, 71).

Similar conclusions as to the importance of acetic acid may be reached from a study of the butyric acid and butyl-alcohol fermentations. Fitz (17, 18, 19) found that butyric acid is produced from glucose, and also from glycerol and lactate, being one of the first to recognize that a synthesis was involved. Acetaldehyde was postulated as an intermediate stage by Buchner and Meisenheimer (8) and Neuberg and Arinstein (58), by means of sulphite, were able to identify acetaldehyde as an intermediate product in butyric fermentations of sugar. From pyruvate they obtained no acetaldehyde, only acetic acid, and no butyric acid was formed. Using a peptone medium containing the decarboxylated anhydride of pyruvic aldol, $\begin{array}{l}CH_3.COH.CO,\\ \quad\ |\qquad\ |\\ \quad CH_2\,.CO\end{array}$ they found that this compound was changed to butyric acid in large amounts. Consequently they formulate the production of butyric acid and butyl-alcohol from sugar, glycerol, or lactic acid, as occurring *via* pyruvic acid, of which 2 molecules go to pyruvic acid. If this suggestion were correct, it would be surprising that the organisms produce no butyric acid on a pyruvate medium. Might one not rather suppose that pyruvic acid is broken down to acetic and formic acids, and the latter is decomposed to hydrogen and carbon-dioxide so rapidly that no hydrogen remains to reduce such acetoacetic acid as may be formed to butyric acid? It is noteworthy that acetone (and so acetoacetic acid) can be produced from pyruvate by the acetone-forming bacteria. When the butyric acid bacteria are growing on lactic acid, hydrogen would be available from the lactic acid for reduction of acetoacetic acid. The production of butyric acid from "pyruvic aldol" is possible without reduction, by loss of CO_2 and addition of the elements of water, as formulated by Neuberg and Arinstein:



$$\begin{array}{l}CH_3.COH.CO\\ \ |\qquad\ \ |\\ CH_2.CO\end{array} \longrightarrow \begin{array}{l}CH_3.CH_2\\ \qquad |\\ \quad CH = CO\\ + CO_2\end{array} + HOH \longrightarrow \begin{array}{l}CH_3.CH_2\\ \quad |\\ CH_2.COOH\end{array}$$

Further information on butyric fermentation is available in the papers of Speakman (84), and Reilly, Hickinbottom, Henley and Thaysen (80). These workers were using organisms which produced from starch butyl-alcohol, butyric acid, acetone, ethyl-alcohol, acetic acid, hydrogen, and carbon-dioxide. Butyl-alcohol and acetone arose late in the fermentation, at the same time as there was observed a fall in the amounts of butyric and acetic acids. The addition of acetic acid gave a noteworthy increase of acetone (Speakman, 84); propionic and butyric acids gave the corresponding alcohols, and therewith an increased yield of acetone, one may surmise from acetic acid, which would otherwise have been reduced to ethyl-alcohol by the hydrogen which reduced the added fatty acid to alcohol. That the butyric acid arose from acetic acid is suggested by the figures given by Reilly and his collaborators for the ratio of acetic to butyric acid. Acetic acid is present almost alone in the early stages; acetic and butyric are present in the ratio of 1 to 1·25 at the moment when formation of butyl-alcohol and acetone begins; after this there is a fall in the relative amount of butyric. These authors report the recovery as acetone of at least 72% of added acetic acid. They postulate the formation of acetone from acetic acid via aceto-acetic acid, and they found that addition of acetoacetic ester to a fermentation led to an increased yield of acetone. They do not suggest that acetoacetic acid may be reduced to butyric acid, but this appears a likely sequel. The occurrence of higher fatty acids in a butyric fermentation has been recorded by Neuberg and Arinstein (58). The marked effect of acetic acid in increasing the fat content of the Timothy Grass bacillus may also be recalled (86). Is it by a path of simple condensation that acetic acid is built up into the long chains of the higher fatty acids?

$$\begin{aligned} &CH_3.COOH + CH_3.COOH \rightarrow CH_3.CO.CH_2.COOH + CH_3.COOH \\ &\qquad \rightarrow CH_3.CO.CH_2.CO.CH_2.COOH, \text{ etc.} \end{aligned}$$

On the views of Quastel (73), the first effect on a saturated fatty acid chain of an activating mechanism, such as is found in the living cell, is the production of an activated molecule of unsaturated type:

$$\underset{\text{Normal}}{—CH_2—CH_2} \rightarrow \underset{\text{Activated}}{—\overset{\vee}{C}—CH_2—} + 2H$$

The product of oxidation of the activated form may be —CH = CH—, —CHOH—CH$_2$, or —CO—CH$_2$— (Quastel, 73, p. 185. These groups may be formed simultaneously and exist in equilibrium. Leathes has shown that extensive desaturation of fatty acid chains occurs in the liver. On Quastel's view, such unsaturated linkages would occur in the β-position to the carboxyl group, and might exist in equilibrium with keto groups. Simple hydrolysis at α-keto group to give acetic acid is usually assumed to occur during β-oxidation of fats. Wakeman and Dakin (93), working on the decomposition of β-oxybutyric and acetoacetic acids by liver enzymes, obtained incomplete evidence pointing to an increase of acetic acid after disappearance of acetoacetic acid.

In synthesis, one might imagine the converse reactions, namely, establishment of equilibrium between keto groups arising by condensation of acetic acid, and the corresponding unsaturated groupings; reduction of unsaturated to saturated compounds, and removal of saturated compounds by synthesis into neutral fats. The necessary material for formation of fat would be accumulation of acetic acid, and available hydrogen (e.g. formic acid). Both these are at hand where rapid breakdown of carbohydrate is occurring, and it seems that the necessary transfer of hydrogen can be effected by the liver. Of interest in this connection are the experiments of Lublin (41), showing that R.Q.'s above 1·0 are consistently found after administration of insulin and sugar to the normal man, i.e., rapid oxidation of carbohydrate, rather than presence of excess, favours conversion into fat.

The Oxidation of Acetic Acid.

One further point may be quoted from Lusk's paper. He speaks of the possibility that "micro-explosions" of acetic acid arising from fat by β-oxidation may be responsible for the energy required in the recovery process of muscle. On his view, under normal conditions both methyl-glyoxal and fat may be oxidized *via* acetic acid to provide this energy; in starvation or diabetic conditions fat may be the sole fuel. It is, of course, obvious, though perhaps still worth mentioning, that the R.Q. of acetic acid is unity. The discrepancy between the R.Q. of muscular work and the total R.Q. of the organism during carbohydrate inanition could be explained by assuming local combustion of acetic acid, after desaturation and β-oxidation of fats in the liver. Liver R.Q.'s of the necessary low values have been recorded by Loeb (40); the R.Q. of a fatty liver from a phloridzinized animal was as low as 0·2. On this view the necessity of assuming any extensive formation of carbohydrate from fat would be removed, and, though this process may occur, the evidence indicates that it must be at best a very slow and difficult one.

The conclusions of Raper and Smith (79) are noteworthy. They are led by their work on insulin and the production of acetone bodies by the perfused liver to the following position: "In the presence of adequate supplies of glycogen, only a minimal amount of fat is utilized for supplying the body's needs, and the acetone bodies produced from this small amount of fat are readily oxidized. When, however, the glycogen content of the liver is reduced to a low value . . . fat is called up from the depots, and, accumulating in the liver, is oxidized more abundantly. Over-production of acetone bodies results. . . . In this sense, it may be said that the liver has two types of metabolism at its disposal, which may be termed the carbohydrate type and the fat type, depending on the preponderance of one or the other reserve substance." Or, in other words, the liver has two sources of acetic acid, which comes to appear as the main fuel of the body. One is inclined to allot to it the place others have assigned to pyruvic acid—that of central meeting point of the metabolism of proteins, carbohydrates, and fats. From fats and some amino acids it arises by β-oxidation of the fatty chain, from carbohydrates by oxidation of acetaldehyde, and from the sugar-forming amino-acids, either via sugar or possibly by breakdown of pyruvic acid. From acetic acid fat synthesis begins, and probably glycine formation; that it may be rebuilt into carbohydrate seems probable for yeast (Meyerhof, 50), and for bacteria which can use acetic acid as sole source of carbon, but for the animal body this is not proven.

Nothing is known as to the mode of oxidation of acetic acid by the living cell, a matter that now becomes of the keenest interest. Possible intermediate substance, such as glycollic and glyoxylic acids, are less readily oxidized than acetic acid itself. Reilly and his collaborators (80) have speculated on the existence of acetic acid in the reactive form $CH_2 = CO$. In presence of bacteria and muscle tissue, acetic acid acts towards methylene blue as a hydrogen donator of low intensity (Wishart, 95; Quastel and Whetham, 77, 78). It may conceivably undergo synthesis to succinic acid in some degree; succinic acid arising during fermentations of sugar by *B. coli* is usually supposed to originate in this manner. Oppenheimer (*Die Fermente*) suggests that acetic acid may be metabolized via succinic, oxalacetic, and pyruvic acids, back to acetaldehyde. It would seem that the oxidation of acetic acid to carbon-dioxide and water must represent two-thirds or more of the energy supply of the animal body. Anything which might help us to discern the processes involved would illuminate a dark and difficult area at the heart of the subject of biological oxidations.

SUMMARY.

1. The relationship of pyruvic acid and acetaldehyde in bacterial and animal metabolism is considered. Enquiry is made as to the way in which pyruvic acid is broken down by bacteria and by the animal body.

2. The origin of acetaldehyde during the oxidation of carbohydrate is discussed on the basis of a suggestion made by Lusk; reference is also made to the subsequent fate of acetaldehyde.

I am glad to have this opportunity of offering my warmest thanks to Professor Sir F. G. Hopkins, Miss M. Stephenson, and Mr. A. B. Anderson for much help and advice. My thanks are also due to the Medical Research Council for a grant.

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ACHROMOBACTER PEROLENS *(n.sp.)* THE CAUSE OF "MUSTINESS" IN EGGS

by

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Our attention was drawn to the problem of the cause of "mustiness" in eggs by Messrs. Rugg and Clinton, of the Produce Division, Department of Agriculture. Musty eggs have a very characteristic odour, which cannot be masked by pastrycooks in the ordinary manufacturing processes. The presence of such eggs in a mixture may, therefore, lead to the loss of much valuable food.

An egg was received on June 11, 1926. It had come from a cool store, and was stated to be a typical "musty"egg. It possessed a peculiar characteristic smell, which was very penetrating, and persisted in the cupboard, in which the egg had been placed overnight, for several days. The egg was sterilized externally with alcohol, and was broken carefully into a sterile Petri dish. A very strong odour was observed. The yolk appeared quite normal and the yolk membrane strong, but the white of the egg showed a definite opalescence suggestive of bacterial contamination. Examination of stained smears made direct from the egg-white showed numerous small gram negative, somewhat bipolar cocco-bacilli, apparently present in a pure state. Slopes of nutrient agar and other ordinary culture media were inoculated with the egg-white and placed in the incubator at 37°C. No growth was apparent after several days. The tubes of inoculated media were then removed to a cupboard at room temperature, and after twenty-four hours very small, round, semi-translucent colonies became apparent. They increased in size in the next twenty-four hours, and a strong musty odour was observed. The micro-organism was apparently present in pure culture, but single colonies were selected and replated to ensure purity. The following are the characteristics that have been observed.

(*a*) Morphology and Staining Reactions. The micro-organism is small, and varies in shape from that of an imperfect sphere to that of a cocco-bacillus, with occasional longer, round-ended, bacillary forms showing in the cultures. It does not readily form chains, but a few short chains may be met with in broth cultures. Its width is fairly constant. The limits of size in broth are from $0{\cdot}4\mu$

to 2·55μ long by 0·35μ wide, when stained with toluidine blue. On agar its size tends to remain more constant, and varies from 1·2μ to 1·4μ long by 0·3μ wide. It is aerobic, not growing in media under a vaseline seal; does not form spores; is gram negative, non-acid fast, and stains well with the ordinary anilin dyes, though the bulk of its staining material is concentrated at each end, so that stained specimens show a bipolar appearance. It is quite motile, and when stained by the simple and excellent method of T. Inouye, is seen to possess a single polar flagellum, usually with two undulations. When thus stained, the average size of organism from agar slopes is 1·6μ long by 0·7μ wide, while the flagellum is from 5μ to 6μ long.

(*b*) Physiology. The temperature limits have not been ascertained, but it has been determined that the organism will not grow at 37°C, which suggests that it cannot be an inhabitant of the body of the fowl; but it grows well at room temperatures, and grows fairly well, though slowly, in the ice-chest. On all media it soon produces its characteristic odour, though the intensity of the smell varies in degree on different media. Sown into sugar-free litmus, Lemco broth, and Durham tubes, with the addition of 1% of the various carbohydrates and alcohols, the following fermentation reactions were obtained after 14 days at room temperature:

(i) Acid but no gas is produced in glucose, levulose, galactose, glycerin, mannit, arabinose.

(ii) No change is produced in saccharose, maltose, lactose, raffinose, dulcit, salacin, inulin.

It does not produce indol (Ehrlich's test). Nitrates are reduced within 24 hours to nitrites, which quickly become reduced to ammonia. Litmus is slowly reduced.

(*c*) Cultural characteristics. After 72 hours at room temperature:

Agar Stroke	Moderate growth; whitish by reflected and semi-translucent to transmitted light. The growth is glistening, somewhat raised along the course of the stroke, but spreads as a transparent flat growth of about ⅛ in. to ¼ in. from the stroke, with finely undulant border. It has a butyrous consistency, and the medium is not coloured. Typical odour very strong.
Broth	A good turbid growth, with slight pellicle and a flocculent sediment. Typical odour very strong.
Glycerine Agar	As on agar, but growth is slightly less abundant; odour strong.
Martin's Agar with 8% Horse Serum	Abundant, filiform, convex, glistening, smooth, semi-opaque, butyrous growth, with no pigment in the medium.
Inspissated Serum	Liquefaction along the line of growth, with a pale, lemon fluorescent tint of the growth.

Dorset's Egg	Abundant, spreading, raised, glistening, smooth growth, the accumulated growth in the water of condensation having a slight orange colour.
Agar Stab	Growth occurs best at the top, with the line of puncture beaded.
Gelatine Stab	Growth occurs best at the top. The line of puncture is beaded, and crateriform liquefaction occurs. The medium is not coloured.
Litmus Milk	No apparent change.

In Martin's broth with serum the growth is similar to that in ordinary broth, but is heavier when horse serum is used instead of ox serum. No growth takes place under anaerobic conditions, such as in liver broth under vaseline seal. Cooked meat medium shows no change of colour.

Further incubation up to three weeks at room temperature causes some changes in the appearance of the cultures. The agar stroke shows a wider film of growth. The broth becomes fairly clear, with a heavy deposit and a thick pellicle. In Martin's broth with 8% ox serum a faint lemon-yellow fluorescence develops. There is complete digestion of the slope of inspissated serum. There is no increase of growth along the line of inoculation in the agar stab, but the growth spreads over the surface as a glistening, circular disc with a thicker, cribiform centre. The growth on glycerine agar is heavier than on plain agar, and the centre raised track is finely wrinkled. The gelatine stab shows an even liquefaction to a depth of 2 cm., with a heavy deposit in the liquefied gelatine. There is very little growth along the track below this level. On potato there is a fairly thick, glistening, raised growth with a brownish tinge. Litmus milk turns red, with gradual decolourization of the litmus and a partial, fine clotting of the caseinogen.

Transmission Experiments.

Fresh, normal eggs were used in the experiments. They were divided into two groups, one being washed and the other being unwashed. The treated eggs were washed well with sand-soap and warm water in an attempt to remove the "bloom," or natural fatty or waxy covering of the egg-shell, which is said to be protective in function.

Two eggs from each group were treated by rubbing a broth culture of the micro-organism over a small area of the shell in each case. They were then placed in a Mason fruit-jar, the atmosphere of which was kept moist by the presence in the jar of moistened cotton wool. The eggs were removed from the jar after six days and examined. They were sterilized externally and broken into sterile Petri dishes. The eggs that had been washed showed distinct opalescence of the white and a more marked odour than the unwashed eggs, the whites of which were not distinctly opalescent. Cultural examination showed a pure growth of the micro-organism, with typical odour, but there were fewer colonies developing from the white of the untreated than from the washed eggs.

In another experiment two eggs from each group were placed in a jar of broth inoculated with the organism, and were examined after six days, with results similar to those obtained in the previous experiment. It thus appears that this flagellated organism can penetrate through the shell of the egg and infect the contents.

Since the first musty egg was examined another has been received from a different source. From this an organism, apparently similar to, if not identical with, that obtained in the first case, has been isolated.

Under the classification adopted by the American Society of Bacteriologists the organism responsible for "mustiness" in eggs belongs to Family 4—Bacteriaceae; Tribe 2—Achromobactereae; Genus 5—Achromobacter, which are characterized as non-pigment-forming (at most no pigment formed on agar or gelatin) rods occurring in water and soil; motile or non-motile; gram negative. The type species is *Achromobacter liquefaciens* (Frankland).

The organism now described most closely resembles in general characters *Achromobacter liquidum,* but this latter organism does not change litmus milk, and has a thick, flesh-coloured, rough, moist growth on potato. In addition the "musty egg" organism is quite characteristic in the odour which it produces. On account of this characteristic and penetrating, musty odour, the specific name *perolens* (L. perolere, to emit a penetrating odour), appears appropriate for the organism. The name proposed for the organism is, therefore, *Achromobacter perolens.*

SUMMARY.

1. Mustiness in eggs is caused by the infection of the eggs with a specific organism, *Achromobacter perolens* (n. sp.).

2. The possibility of eggs becoming infected in the body of the fowl is remote, since the organism will not grow at the temperature of the fowl's body.

3. Since the organism grows at room temperature it is probable that infection takes place after the egg is laid.

4. As the organism will grow slowly at or slightly above 0°C., it is possible that it may infect eggs in the cool stores.

Experiments to determine this are indicated.

OBSERVATIONS ON THE LARVAE OF ONCHOCERCA GIBSONI (CLELAND AND JOHNSTON) IN THE SKIN OF INFECTED CATTLE

by

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INTRODUCTION.

This note records the finding of larvae resembling the microfilariae of *Onchocerca gibsoni* in the superficial layers of the dermis of most parts of the body of cattle bearing nodules of *Onchocerca gibsoni* in the brisket.

It must be pointed out, however, that while in the cattle examined, typical nodules containing living worms and larvae of *Onchocerca gibsoni* were present, the presence of other species of *Onchocerca* which, as recorded by Johnston (1921), may occur in Queensland cattle, was not excluded. These species are *O. gutturosa* Neumann and *O. lienalis* Stiles.

Onchocerca gibsoni, described and named by Cleland and Johnston in 1910, has been the subject of extended investigations from most points of view by these and a number of other observers in Australia. To attempt to even summarize this work would be beyond the scope of the present note, and unnecessary, since reviews of it have been published from time to time by Johnston. Only those observations will be referred to which bear more directly on those here recorded. The most recent paper is that of Cilento (1923).

The work in Australia has established the fact that the larvae are either absent or very scarce in the blood. The same is generally true of the closely related parasite *Onchocerca volvulus*, although occasional larvae have been found in the blood by several observers, often probably derived in reality from other tissue. Sharp (1926) has described an interesting but exceptional case, where these microfilariae were constantly present in the blood. But although the microfilariae of *O. volvulus* are absent from the circulating blood of infected African natives, they are present in the dermis outside the blood vessels in considerable numbers. It was this fact, noted by Montpellier and Lacroix (1920), Macfie and Corson (1922), and others, subsequently to most of the Australian work on *O. gibsoni*, which suggested the present observations.

The presence of the larvae of *O. volvulus* in the skin often gives rise to no lesions or macroscopic signs of their presence, although some observers maintain that certain skin affections are caused by them.

More recently Blacklock (1926) has shown that *O. volvulus* is transmitted by *Simulium damnosum*, which abrades the skin in the act of biting, and takes up other material from it besides blood.

Cleland (1914) found larvae of *O. gibsoni* in small thickened patches of the skin of cattle on two occasions, and published photographs of sections of this skin showing the larvae *in situ*. He also found several larvae in the subcutaneous tissues of cattle. It was not at that time recognized, however, that the larvae are present in the skin of the body generally, and in much larger numbers than in other tissues, in which presumably those that are found are merely making their way to the skin.

DETAILS OF OBSERVATIONS.

In July, 1926, a slaughter-yard in Townsville was visited, and a preliminary examination made. Three beasts, all of which had typical nodules of *Onchocerca gibsoni* in the brisket, were examined. A piece of skin, 2 or 3 inches in diameter, was taken from this region of two of the beasts, and two pieces from the third, and in all four specimens larvae were found by immersing the minced skin in physiological saline at room temperature for twenty-four hours and then centrifuging. The larvae seemed identical in appearance with those obtained from nodules, and agreed with the published descriptions of the larvae of *O. gibsoni*.

In these and the further experiments to be detailed below the animals were examined immediately after killing and before skinning. As soon as the presence of nodules was ascertained by palpation and incision the selected pieces of skin were shaved as rapidly as possible and dissected off.

At a second visit to the yard shortly afterwards one beast only was examined, a bullock with typical nodules in the brisket; they were not looked for in other regions. The procedure adopted was as follows: Immediately after killing, small pieces of skin were first roughly shaved, and then dissected off from the different parts of the body detailed in the table given below. This bullock is hereinafter referred to as bullock 1. From each piece of skin twelve circular pieces were punched out by means of a leather punch, and placed in a test tube containing about an inch of physiological saline solution. This was done at the slaughter-yard with as little delay as possible. The diameter of each circular piece of skin punched out was exactly 4 mm., which gives a total area of 150 sq. mm. for twelve pieces. The test tubes were taken to the laboratory, and left at room temperature for twenty-four hours, after which the pieces of skin in each tube were removed from the salt solution, shaken with fresh solution, and the whole of the fluid centrifuged. After removal of the supernatant fluid with a pipette, the deposit left was well mixed, and a part of it pipetted on to a slide, mixed with a little serum to make it adhere, spread out as a thick layer, and allowed to dry. It was then fixed in rectified spirit, dried, stained with Mayer's haematein, washed and dried, mounted in cedarwood oil, and the larvae counted. The number of larvae obtained from the samples of skin from different parts of the body are given in the table below. In this case the numbers given in the table are the actual numbers counted multiplied by three, since a third was judged roughly to be about the fraction of the deposit examined.

At a third visit to the slaughter-yard, in August, 1926, samples of skin and other parts were again taken from a single bullock in the same manner as before. The work was begun immediately the beast was killed, and all the specimens had been removed within an hour and a half of death; all the pieces of skin within half an hour. Typical nodules were present, as before, in the brisket, and were not looked for elsewhere. Twelve circular pieces of the same size as before were punched from each piece of skin and mucous membrane, and treated in the same way. This bullock is referred to as bullock 2. In this case nearly all the deposit got by centrifuging was examined, so that the number of larvae actually counted approximated to the total number which had emerged from the little discs of skin.

Region of the Sample of Skin or Mucous Membrane.	Number of Larvae obtained from Bullock 1.	Bullock 2.
About 6 inches from the mid-ventral line of the chest, just posterior to the foreleg	327	105
Base of neck, near mid-ventral line	525	235
Over the vertebral column about the level of the fore limb	231	978
Over the vertebral column, about midway between fore and hind limbs	219	91
A point about half-way between the root of the tail and the great trochanter	48	5
Lateral aspect of the thorax, about midway between the fore and hind limbs	15	20
Abdomen, a little to one side of the mid-ventral line, and rather nearer to the pubic symphysis than to the costal margin..	87	684
Mid-ventral line between scrotum and tail	6	103
The scrotum	Not examined	71
Dorsal surface of the tail, about a quarter of its length from the root	0	2
Under surface of the tail, about 8 inches from the root	Not examined	7
Forehead, between the eyes	3	7

Region of the Sample of Skin or Mucous Membrane.	Number of Larvae obtained from Bullock 1.	Bullock 2.
Dorsum of nose, half-way between the end of the snout and the eyes	12	1
Outer surface of the pinna	Not examined	1
Inner hairless surface of the pinna	Not examined	4
Outer aspect of the forearm	87	24
Inner aspect of the forearm	Not examined	49
Outer aspect of the carpus	120	94
Inner aspect of the carpus	Not examined	109
Outer aspect of the leg, in the sense of the section of the hind limb supported by the tibia	48	9
Inner aspect of the leg	Not examined	9
Outer aspect of the tarsus	108	33
Inner aspect of the tarsus	Not examined	26
Inferior margin of the nostril just within the junction of the skin and mucous surface	Not examined	60
Mucous membrane of the inner surface of the cheek	Not examined	0
Mucous membrane of the side of the tongue	Not examined	0

Although the figures in this table are considered worth recording, they probably give somewhat imperfect indications of the distribution of the larvae in either beast. The samples were taken from bullock 1 merely with the idea of finding out whether the distribution of the larvae was general over the skin of the whole body. Bullock 2 was intended to be the first of a series to determine the average distribution more exactly with the idea that it might show a relation to the regions most favoured by some of the biting flies. The series could not be continued, and the method would have required improvement. The ratio of the number of larvae which emerged into the saline to the total number in the twelve discs of skin very likely differed considerably for the different samples, for various reasons.

It will be seen, however, that while no close agreement was found between the distribution of the larvae in the skin in the two bullocks, yet in general, with a few exceptions, the regions of abundance and scarcity correspond.

Some observations indicated that the larvae probably emerged from the cut edges of the discs of skin rather than through the epidermis, or by working their way down through the whole thickness of the dermis. In bullock 2 four roughly rectangular pieces of skin, two from the base of the neck and two from the front of the thorax, were cut off from the same two samples from which the punches were made. The perimeter of each of these four pieces was approx. 150 mm., which is also the total perimeter of twelve discs 4 mm. in diameter; on the other hand, the area of each was about 1,500 sq. mm., which is far greater than the area of the twelve discs, 150 sq. mm. Each of these four pieces of skin was immersed whole in saline in a petri dish, and the number of larvae obtained after twenty-four hours was of the same order as the number from the twelve discs, namely, 355 and 169 from the two pieces from the neck, and 203 and 106 from the two pieces from the thorax.

Trial was not made whether any larvae at all would emerge through the epidermis or through the thickness of the dermis into saline or water in contact only with intact portions of these surfaces, nor were the larvae directly observed in the act of emerging, which could readily be done. Breinl's (1911) results with living beasts, in which larvae were found in water kept in contact with the shaved skin near nodules or in scrapings of the skin, in five experiments out of a hundred, were presumably due to the emergence of larvae lying in the dermis, possibly as a result of the stimulus of shaving or scraping, and perhaps through microscopic breaches in the epidermis caused by these procedures.

It may possibly be that any trauma of the skin, by a biting fly or otherwise, acts as a stimulus to neighbouring larvae to make towards the point, and thus increases their chances of being ingested.

A simple method of ascertaining whether larvae are present in the skin, and gauging their numbers roughly, which has been used for *O. volvulus* in African natives, would probably be of service in living cattle. It consists in pinching up a piece of skin, shaving off a small piece of epidermis with a sharp knife, and then scraping off the tissue fluid mixed with blood which exudes, and transferring it to a slide.

It is possible that an investigation by this method might reveal that, as in the case of *O. volvulus* in man, the percentage of cattle with larvae in the skin is much larger than that with discoverable nodules.

In bullock 2 a piece of the sample of skin removed from the base of the neck was dropped about twenty minutes after removal into picro-sublimate fixing solution, and subsequently serial sections were made, a difficult procedure in skin of this character, but accomplished by Mr. J. W. Fielding, of this Institute. The sections were cut 22 microns in thickness, and stained with Mayer's haematein. Thick sections, provided that a purely nuclear stain is used, give the best view of the larvae; even in sections of this thickness a complete uncut larva was only once seen. It must be emphasized that the brief account which follows of the appearances in these sections may not be true for the living animal in every particular, owing to the possibility of movements of the larvae in the interval between the death of the animal and their killing by the fixative. It would be better to remove the piece of skin the moment the beast has been pithed and plunge it into hot fixative. In the sections the microfilariae were usually more or less coiled, but sometimes extended. They were present only in the superficial layers of the dermis, which is very thick in this part of the body; the deepest noted was about 0·6 mm. from the basal layer of the epidermis. Often they were immediately beneath the epidermis, and were then perhaps more often at the summits of dermal papillae than in the valleys between papillae. Commonly enough, however, they were some distance beneath the epidermis, and were then often close to the sheaths of hairs or to sebaceous glands. No larvae were seen within the epidermis. The only changes noted in the skin tissues were slight aggregations of cells in the superficial layers of the dermis round the capillaries and small blood vessels. In sections stained with eosin as well as haematein these were seen to include many eosinophile leucocytes.

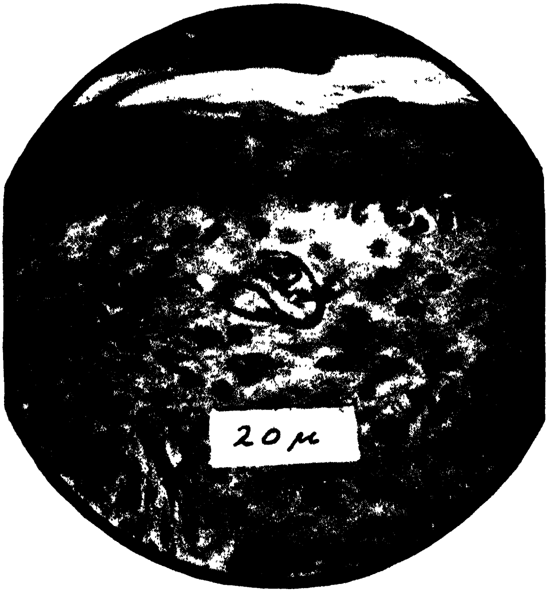
It is of interest in connection with the mode of transmission to know the number of larvae present beneath a given area of skin, and an attempt was made to determine this in the particular specimen from which serial sections were made. Various methods were tried, of which the simplest was to count the number either of tails or of heads present in a series of sections. It was eventually found that the number lay between 15 and 40 per square millimetre of this skin. As only 235 larvae emerged from the twelve punched pieces of this sample of skin, that is, about 1·6 per square millimetre, it is evident that most of the larvae do not emerge under the conditions of the experiment from discs of skin 4 mm. in diameter. Probably by mincing the skin more finely a much larger proportion of the total would be obtained.

In bullock 2 some of the other tissues and organs besides the skin were examined. About 10 grm. of the tissue, which was not obtained until after the animal had been skinned and partly cut up, was minced with a knife and placed in a large test tube with some physiological saline solution. On arrival at the laboratory the contents of the tube were transferred to a petri dish and more saline added; this was centrifuged after twenty-fours hours, and the larvae in the deposit counted. The findings were as follows:

1. Superficial fat of the brisket taken quite close to nodules (only about one-third of the centrifuge deposit examined), two larvae found.
2. Superficial fat further forwards at the base of the neck, not far distant from nodules (only about one-third of the centrifuge deposit examined), two larvae found.
3. Muscle from the brisket taken quite close to nodules, one doubtful larva.
4. Muscle of forearm, no larvae found.
5. Liver (only a fraction of the centrifuge deposit was examined, owing to its bulk), no larvae found.
6. Lung, containing but little blood, three larvae found.
7. Heart muscle, ventricle, no larvae found.

DISCUSSION.

The Vector. No discussion of this question, on which so much careful experimental work has been done in Australia, will be attempted here beyond one or two remarks.



Photograph of a larva in a section of skin from the base of the neck of Bullock 2.

The solution of the problem for *O. volvulus* of course increases the probability that a dipterous insect is the vector of *O. gibsoni*. This fly must be one which imbibes not only blood but tissue fluid. For this reason it seems unlikely that a mosquito is concerned, since its slender and delicately wielded piercing organs cause the minimum of laceration and abrasion, and possibly are inserted directly into a small vessel.

The inference from the experiments of Hill, McEachran, and Dickinson (1917) that the vector, if a biting fly, is one which keeps to the open, and does not bite even in an open shed, appears noteworthy.

The Cycle in the Mammalian Host. Nicholl (1914) has shown that larvae can escape through the intact fibrous wall of excised nodules, and doubtless they do so during life. But in the case of *O. volvulus* the larvae are present in the skin of many natives in whom no nodules can be found, and Sharp (1926) has suggested that the formation of nodules is a late phenomenon, and that mature and fertile worms often live free in the tissues. The same suggestion had previously been made by Cleland concerning *O. gibsoni*, but attempts to find the uncapsuled worms have met with little success.

That the route of the microfilariae from the parent worm to the skin is not the blood stream is made probable by the numerous unsuccessful attempts to find them in the blood, while on the other hand a few have been found in subcutaneous tissues on several occasions by Cleland (1914), and in sections of tissues outside blood vessels by Breinl (1913). The fat near nodules in which, as recorded above, a few larvae were found contained very little blood.

SUMMARY.

The distribution of larvae throughout the skin of cattle infested with *Onchocerca gibsoni* is described, and the similarity to the condition found in *Onchocerca volvulus*, in which the method of transmission is known, is pointed out.

In the cattle observed, however, the presence of other species of *Onchocerca* was not excluded.

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MITOCHONDRIAL BEHAVIOUR DURING THE LIFE CYCLE OF NYCTOTHERUS CORDIFORMIS

by

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(Submitted for publication 27th April, 1927.)

Recently the presence of mitochondria in protozoa has attracted a great deal of attention among cytologists, and it is the object of this paper to give an account of a further series of observations made upon the mitochondria of a common infusorian, Nyctotherus, and to describe the changes which they undergo during the life cycle of that organism, and the part mitochondria appear to play in cell metabolism.

Methods of Investigation.

In order to observe the mitochondrial behaviour during the life cycle of this infusorian, the rectal contents of starved frogs *(Hyla aurea)*, which are generally infested with *N. cordiformis*, were fixed in Flemming solution without acetic acid, or else in Champy fixative. The organisms were concentrated by centrifuging, and eventually embedded *en masse* in paraffin, and then finally sectioned. These sections, which were cut 4–6μ in thickness, were stained with Heidenhain's iron haematoxylin, and occasionally counterstained in eosin.

On examining these sections with high magnification, the cytoplasm of the protozoan is seen to contain a very large number of rod-shaped bodies, which frequently lie in little groups, having a bacteria-like appearance; but their subsequent behaviour and reactions to certain stains and fixatives show quite clearly that they cannot be symbiotic organisms. When a number of individuals were fixed in absolute alcohol, instead of in osmo-chromic or Champy solutions, these bodies were incapable of being stained by iron-haematoxylin. In this respect they resemble the mitochondria present in other protozoan and metazoan cells, in which these bodies are rendered non-stainable by fixatives containing alcohol. On the other hand, during my previous observations on mitochondria, I found that if various strains of bacteria were fixed for several hours in absolute alcohol, their capacity for being stained with iron-haematoxylin was not visibly affected by this mode of fixation; while mitochondria placed for the same period in absolute alcohol were quite incapable of absorbing the haematoxylin stain (see figure 5). The non-bacterial nature of the bodies dealt with in this paper is also demonstrated by the following facts: If Nyctotherus be placed for a short time in Ringer's solution, and afterwards sectioned and stained, examination shows that the cytoplasm of the organism contains an increased number of mitochondria (see figure 6), which have evidently been formed by fission of the mitochondria of the normal organism. During my previous observations on Opalina (4), I observed that a similar phenomenon occurred, the Ringer's solution apparently acting upon the mitochondria, stimulating them to increase in numbers, by means of binary fission, the mitochondria subsequently undergoing fusion in places to form large, darkly staining globules. Similar post-mortem effects may be observed with the mitochondria of metazoa (4), merely by allowing the tissue to stand for a short period before fixation. We are justified in concluding, therefore, that these bacteria-like, rod-shaped cytoplasmic inclusions of Nyctotherus are of the same nature as the mitochondria present in other protozoan and metazoan cells.

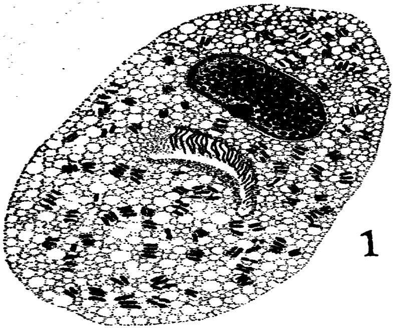
Behaviour of the Mitochondria.

When the adult Nyctotherus, cut in longitudinal sections, and stained with the Heidenhain process, is examined under high magnifications, the cytoplasm is seen to contain numerous rod-like bodies. If the outer region of the organism is observed, it is seen that these bodies are arranged one behind the other in longitudinal rows, the individual mitochondria attaining a transverse polarity to the axis of the cell (see figure 2); if, however, the deeper parts of the cytoplasm are examined, these mitochondrial bodies are seen to be irregularly scattered throughout the protoplasm (see figure 1).

DESCRIPTION OF PLATE.

All figures (except figure 5) are drawn from material fixed in Osmo-chromic or Champy solutions, and stained with Heidenhain's iron-haematoxylin.

1. Longitudinal section of an adult Nyctotherus, showing the arrangement of mitochondria. The meganucleus and micronucleus and gullet are clearly visible.
2. Oblique section of an adult Nyctotherus, showing the arrangement of the superficial mitochondria, in longitudinal rows. Note the irregular arrangement of mitochondria in the deeper parts of the organism.
3. Longitudinal section of the same, showing three food vacuoles containing mitochondria (not bacteria).
4. Section of Nyctotherus undergoing longitudinal fission. Note the more or less equal distribution of mitochondria into the two developing daughter cells. It is clearly seen that the division of the cell is not accompanied by fission of the mitochondria.
5. Longitudinal section of Nyctotherus, after fixation in absolute alcohol, showing how the mitochondria are incapable of being stained by iron-haematoxylin, after such mode of fixation. Note deeply staining meganucleus.
6. Section of same, showing increase in number of mitochondria after organism has been placed in Ringer's solution. Many mitochondria can be seen undergoing fission.



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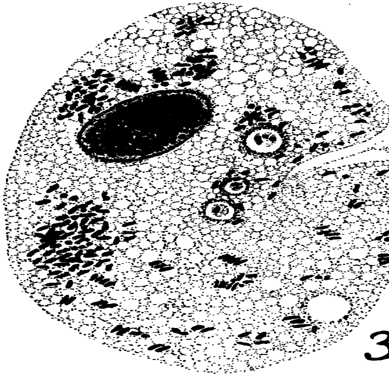

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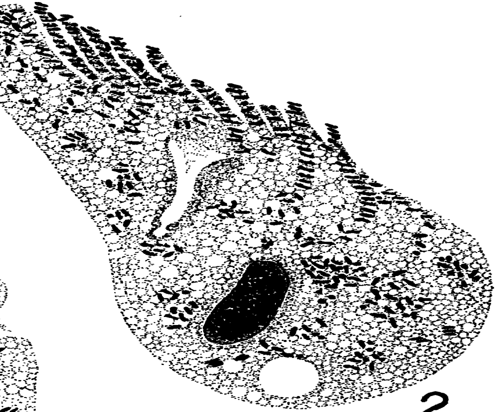

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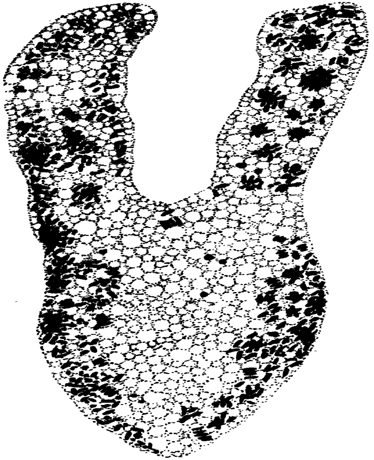

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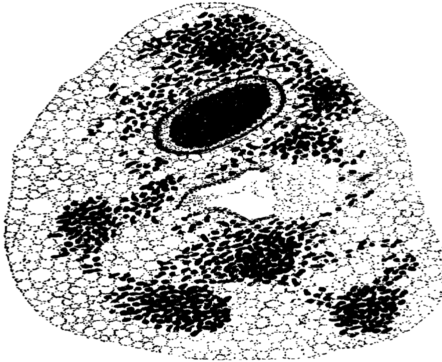

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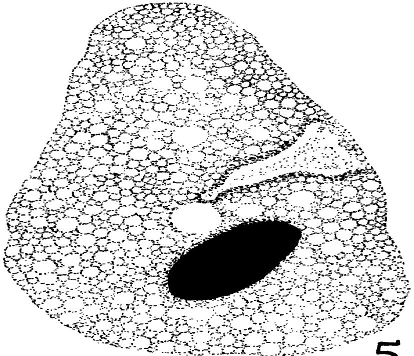

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A further examination of the mitochondria present in both the adult and daughter cells shows that the mitochondria increase by a transverse binary fission, and moreover that fission occurs at irregular intervals. Fauré-Fremiet (2), in some of his former works dealing with mitochondria in the protozoa, states that division of the cell is accompanied by a simultaneous fission of the mitochondria. But I have previously found that in Opalina (4) and in Paramoecium (5) no such phenomenon occurs. I am now able to report that in Nyctotherus also mitochondrial division does not synchronize with nuclear fission (see figure 4). As in Paramoecium and Opalina, so I find that in Nyctotherus there occurs a gradual increase in their number during the growth of the individual, and the easily observed fact that the number of mitochondria present in the young daughter cells is far less than the number present in the adult cells does not add weight to the statement of Fauré-Fremiet.[1]

The Function of Mitochondria in Cell Metabolism.

Quite recently Marston (8) has shown that the behaviour of the mitochondria towards azine dyes is exactly identical with the reactions which the proteolytic enzymes undergo in the presence of these substances, and he is led to the conclusion that they contain concentrated within them the synthesizing enzymes of the cell, their action being to build up protein at their surface.

In earlier years Regaud and Mawas (9) suggested that in the salivary glands the secretory granules arose from the mitochondria, and pointed out their relationship to enzymes; while Arnold (1) in 1912 showed that the zymogen grains in the pancreas arose from cell mitochondria. I have also observed (6) that the zymogen granules present in the pancreas of guinea-pigs arose from the filamentous mitochondria, and were secreted into the acinar ducts, where they eventually became dissolved. These observations strongly suggest the enzymatic nature of the mitochondria.

Later observations have shown that in Paramoecium (5) and in Amoeba (7) the mitochondria come into contact with the food particles within the food vacuoles, and apparently bring about disintegration of the food.

Here we seem to observe a process of intracellular secretion of enzymes in organisms which exhibit only intracellular digestion, similar to the process of extracellular secretion as observed in gland cells. Furthermore, in Opalina (4) I have observed that a large granule may develop in close connection with each mitochondrium, this granule evidently being a food storage substance, the result of local synthetic activity occurring through the concentration of diffusible foodstuffs that have penetrated the cuticle of the organism.

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[1] It is interesting to observe that Hegner and Taliaferro (3) state the Nyctotherus increases only by means of a transverse binary fission, while two of my cut and stained sections show that these organisms are also capable of reproduction by longitudinal fission (see figure 4).

Studies on Nyctotherus have shown that in this form synthetic activity of the mitochondria cannot be detected, and it is quite possible that Opalina may be a more favourable object for observing this formation of storage grains.

Nyctotherus, like Paramoecium, feeds in a truly holozoic manner, and a process of intracellular digestion similar to that which I have already described in Paramoecium may be observed also.

Cut and stained sections of Nyctotherus under high magnifications show that rod-like mitochondria, which can easily be distinguished from bacteria, are present in the food vacuoles (see figure 3).

In a recent paper dealing with the function of mitochondria in living amoebae, I observed that when a foreign organism is engulfed by the amoeba, a number of mitochondria adhere to the surface of the food; finally a vacuole is formed around the food particles, and the mitochondria gradually dissolve and the food disintegrates.

In amoebae I have never observed the extrusion of mitochondria into these vacuoles, but the mitochondria always adhere to the surface of the food first, and it is only subsequently that a vacuole is formed around the food particle. In fact, in amoeba there is a good evidence that the mitochondria do not penetrate into the vacuoles, but the vacuole is always formed after the mitochondria have adhered to the captured food.

Living amoebae are particularly favourable objects for the study of this, as the movements undergone by the organism are slow, and permit careful observation.

Nyctotherus, on the contrary, is far too active to permit the making of observations on this question in the living organism. As in Paramoecium, so in Nyctotherus, it must at present be left an open question as to how the mitochondria come to lie within the food vacuoles; of their presence there can, of course, be no doubt, and in making the observations no confusion between mitochondria and engulfed bacteria has arisen. In living amoebae, as I have previously reported (7), mitochondria may lie close to the wall of the vacuoles, where, undergoing marked Brownian movement, they even strongly indent the surface of the vacuole; nevertheless, I have never observed them to break through into it.

In analogy with what we find in amoeba, therefore, it seems more probable that the mitochondria are not thrown into the food vacuoles, but that the vacuoles are only subsequently formed around food particles to which the mitochondria have previously adhered.

SUMMARY.

1. Mitochondria are present in Nyctotherus during all stages of the life cycle; they increase by transverse binary fission at irregular intervals during the life history of the organism, and there is no relation between nuclear division and fission of mitochondria.

2. Mitochondria are found within the food vacuoles of Nyctotherus. This is regarded as a direct demonstration of the origin of digestive enzymes from mitochondria.

I am greatly indebted to both Professor W. E. Agar and to Dr. O. W. Tiegs for their valuable suggestions. This investigation was carried out while holding a Science Research Scholarship in the Department of Zoology of the University of Melbourne.

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ON THE RELATION OF MITOCHONDRIA TO THE NUCLEUS

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INTRODUCTION.

This small piece of work has been carried out in order to investigate the relation of the mitochondria to the nucleus. Various authors have frequently drawn attention to the peculiar aggregation of mitochondria round the outer surface of the nuclear membrane in many cells, while Rikita Honda, in a recent paper (3), states that mitochondria are actually traceable through the nuclear membrane. As this conclusion of Rikita Honda is not in accord with my own observations, I have considered the subject deserving of a further investigation.

MATERIAL AND METHODS.

In order to investigate the phenomena of the mitochondria in the nuclear region, both protozoan and metazoan tissue was used. When dealing with the protozoa, two common types of Infusoria, namely, Nyctotherus and Paramoecium, fixed in either a Flemming solution without acetic acid, or else in a Champy fixative, were concentrated by centrifuging, brought up through the alcohols, cleared, eventually embedded *en masse* in paraffin, and finally sectioned. The sections were cut 3–5μ in thickness, stained with Heidenhain's iron-haematoxylin, and occasionally counterstained with eosin.

The metazoan material used in this research was the submaxillary gland obtained from the adult albino rat. It was treated by exactly the same methods as regards fixation and staining as the protozoan material, the only difference being that the sections were cut somewhat thicker, having a thickness of 6μ.

If prepared sections of either Nyctotherus or Paramoecium are examined under high magnifications, the cytoplasm of the organism is seen to contain a large number of rod-like bodies, and in cases when a section has been cut through the middle of the meganucleus of the organism, these bodies are generally seen to be aggregated round the outer surface of the nuclear membrane, in a manner which will be described more fully in a later portion of the paper. These rod-shaped bodies certainly present a bacteria-like appearance, but their subsequent behaviour to certain stains and fixatives shows quite clearly that they cannot be foreign organisms. A ready test for mitochondria is available, whereby one may distinguish them from ordinary bacteria. If bacteria are exposed for several hours in absolute alcohol they afterwards stain up sharply with iron-haematoxylin, while mitochondria are rendered non-stainable even after quite a short exposure. In the case of Nyctotherus and Paramoecium this test was applied, and the mitochondria present in both these infusorians were rendered non-stainable by the Heidenhain process (see figure 5). In fact, mitochondria are incapable of absorbing the stain if fixed in a solution containing even a small proportion of either absolute alcohol or acetic acid, while bacteria are not visibly affected by this treatment.

If cut and stained sections from the submaxillary gland of the albino rat are examined, numerous long filamentous bodies, together with a large number of small spherical bodies, may be seen in the cytoplasm of the cells with great clearness, and the filamentous bodies may occasionally be seen extending over the nuclear membrane. These bodies, when treated with either absolute alcohol or acetic acid, are also rendered non-stainable; and, in fact, respond in exactly the same way as those other rod-shaped bodies present in both Nyctotherus and Paramoecium. From these considerations I believe these cytoplasmic inclusions to be of the same nature as the mitochondria present in other animal and plant cells.

OBSERVATIONS AND DISCUSSION.

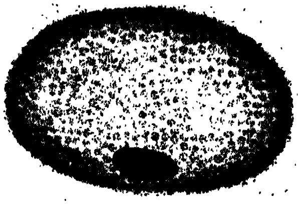
When Nyctotherus and Paramoecium are examined in longitudinal section after staining with the Heidenhain process, the cytoplasm of these organisms is seen to contain a number of minute rod-shaped mitochondria scattered through the cytoplasm.

If, however, a section cut through the middle of the meganucleus be examined, it will be observed that rod-like mitochondria are arranged round the nucleus,

REFERENCE TO PLATE.

All figures (except figure 3) are drawn from material fixed in either Flemming's solution without acetic, or else in a Champy fixative, and stained with Heidenhain's iron-haematoxylin, and occasionally counterstained in eosin.

1. **Oblique longitudinal section, cut through the middle of the meganucleus of Nyctotherus. Note mitochondria arranged around the nucleus, lying quite close to and in intimate contact with outer surface of nuclear membrane. Micronucleus clearly visible.**
2. **Surface section of same, showing mitochondria in close contact with surface of nucleus.**
3. **Absolute alcohol fixation of same demonstrating absence of mitochondria after such treatment. Micronucleus clearly visible.**
4. **Longitudinal section of meganucleus of Nyctotherus, showing a large aggregation of mitochondria in front of and in close contact with outer surface of nucleus.**
5. **Longitudinal section of nucleus from a cell of submaxillary gland, showing filamentous mitochondria lying across outer surface of nuclear membrane.**



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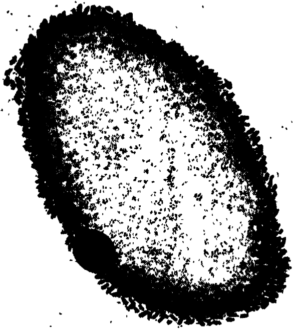

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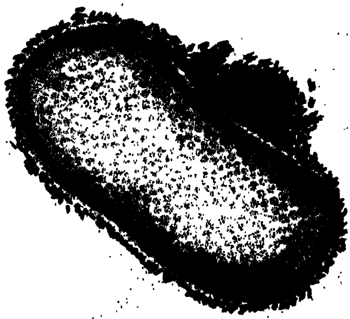

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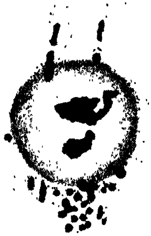

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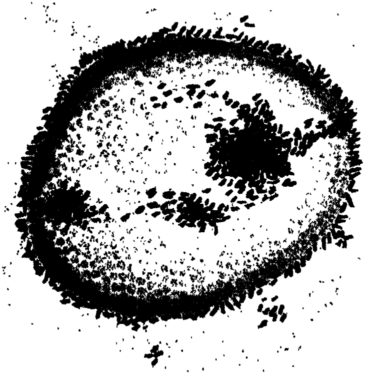

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lying quite close to and apparently in intimate contact with the outer surface of the nuclear membrane (see figure 1).

I further observed that if a surface section of the meganucleus be examined numerous mitochondria were to be seen in close contact with the surface of the nucleus, and often covering the whole surface. This interesting phenomenon may at first give the impression that the mitochondria are actually traceable through the nuclear membrane, but a further and more detailed observation shows quite clearly that these bodies only lie in intimate relation with the outer surface of the membrane, and do not occur within the nucleus (see figure 2).

Rikita Honda, however, in a recent paper dealing with mitochondrial behaviour in the submaxillary gland of the albino rat, states that the mitochondria are actually traceable through the nuclear membrane of the epithelial cells. On reinvestigating this work, I find that stained and cut sections of the submaxillary gland of these rats, prepared by the usual mitochondrial technique, show filamentous mitochondria occasionally lying across the nucleus in close contact with the outer surface of the membrane, in much the same way as do the mitochondria present in both Nyctotherus and Paramoecium. In no case, however, have I observed a similar phenomenon to that described by Rikita Honda, where the mitochondria actually occur within the nucleus.

When examining the acinar cells of the pancreas of the guinea-pig, I have also noticed mitochondria lying in a similar fashion in intimate relation with the outer surface of the nucleus.

Recently many authors have drawn particular attention to the various aggregations of mitochondria which occur in contact with the outer surface of the nuclear membrane in many types of cells. While dealing in particular with Nyctotherus, my attention was drawn to a dense mass of granules frequently occurring in front of and in close relation with the meganucleus (see figure 4). Later I observed that Hegner and Taliaferro (2), as well as Minchin (5) have also remarked upon this fact. The dense granular accumulation may be seen quite clearly as a highly refractive mass in the cytoplasm of the living cell. By applying the usual mitochondrial technique to these bodies, I discovered this granular mass to be an aggregation of mitochondria, presenting a similar appearance to that shown in the figures of Harvey (1) and other authors in various cells, where aggregations of mitochondria collect round the outer surface of the nucleus. Harvey, who made a study of mitochondria in the oogonium of *Lumbricus*, describes and figures a similar dense mitochondrial mass in close contact with the outer surface of the nuclear membrane.

So far as I am aware at present, no author has interpreted the occurrence of this frequent phenomenon. While dealing with this problem it is of interest to note that Mayer, Rathery, and Schaeffer (4) have recently contended that mitochondria are composed largely of phosphatides which contain fatty acid radicles; and moreover, it is known that phosphatides, like other fatty substances, accumulate at the phase boundary of the cytoplasm, owing to their capacity to reduce surface tension, according to the Gibbs-Thomson law. It is possible that the accumulation of mitochondria at the nuclear-cytoplasmic interface may be related to this property of their phosphatidal components. Such a conclusion would also help to explain the frequent occurrence of mitochondria round the vacuolar membranes, and also round the peripheral region of many cells.

SUMMARY.

1. Observations show that mitochondria are not traceable through the nuclear membrane in the epithelial cells of the submaxillary gland, as has previously been stated by Rikita Honda.

2. Dense aggregations of mitochondria about the outer surface of the nucleus, such as is seen in many cells, may possibly be a surface tension phenomenon, and is apparently dependent upon their phosphatidal nature.

This investigation was carried out while holding a Science Research Scholarship in the Department of Zoology of the University of Melbourne.

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THE STRUCTURE OF THE NEURONE JUNCTIONS IN SYMPATHETIC GANGLIA, AND IN THE GANGLIA OF AUERBACH'S PLEXUS

by

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The present investigation is part of an attempt to extend to the remainder of the nervous system the conclusions I have previously reached for the cells of the spinal cord, namely, that the neurone junctions occur, not as discontinuous synapses which are closely applied to, but are not continuous with the body of the nerve cell, or ramify among its dendrites, but that, on the contrary, they penetrate the nerve cell, and become identical with its neurofibrils.

General Remarks.

The minute structure of neurone junctions within the nervous system has been the subject of numerous investigations within the past fifty years. Before the introduction of the special methods of Ehrlich, Golgi, and Cajal, the opinion of Gerlach prevailed, namely, that the central nervous system was composed of a meshwork of nerve cells, the nerve cells anastomosing with one another by way of their dendrites.

With the introduction by Golgi, about 1873, of the silver-chromate impregnation method, the modern neurone doctrine arose. Golgi observed the presence of long fibres within the central nervous system, but declared that there occurred a true neurone continuity, the axon collaterals of one neurone being continuous with the collaterals of other nerve cells.

This theory of collateral continuity was opposed by Cajal, by Forel, by Kölliker, and by Retzius, who concluded that the network of Golgi was in reality merely a feltwork, and that no direct anastomosis of collaterals therefore occurred. On the contrary, it was found, as Arnold had already observed for the frog's sympathetic, in 1865, that the nerve fibres underwent terminal ramifications, which were believed to be applied to the body of other nerve cells. Similar observations by Ehrlich (1886) and others led in 1891 to the formulation by

Waldeyer of the neurone theory, in which the neurones were conceived as the morphological units of which the nervous system was composed. These neurones were not continuous with one another; the endings were by contact—there was not continuity, but contiguity, and to this break in the reflex chain the features by which conduction of a reflex arc is distinguished from conduction along the nerve fibre were attributed. Based on Cajal's conception of the ramifying terminations of nerve fibres embracing the body and dendrites of nerve cells, but not penetrating them, Michael Foster and Sherrington in 1897 proposed the term "synapse" (synapsis), from σὺν, and ἅπτω (clasp).

Although the theory of neurone discontinuity has received the strong support of numerous investigators, Auerbach (3), Semi Meyer (20), Dogiel (10), Retzius (21), Lenhossek, M. Heidenhain (13), and more recently Bartelmez (5), yet there are a number of investigators who have strongly opposed it. In 1897 Apathy (1) described a neurofibril continuity in the nerve cells of invertebrates. Neurofibrils from the sensory neurones communicated with neurofibrils from the motor neurones either directly or by way of an anastomosing neurofibril network (diffuses Elementargitter). This observation was confirmed and extended by Bethe (7). In the leech and earthworm, according to Bethe, neurofibrils emerge from the trunks of the sensory neurones, and either penetrate directly into the motor neurones, or first unite with a small number of other, apparently sensory, neurofibrils to form a "fibrillengitter," from which a single neurofibril emerges, and penetrates the motor cells. While in these worms the indirect passage of neurofibrils via the "fibrillengitter" is believed to occur in the minority only of cases, in the crab *Carcinus* Bethe believes it to be the predominating mode of neurone junction.

Retzius, on the contrary, states that he has examined Apathy's own preparations, and can find no trace of a "fibrillengitter"; there occurs a twist-work, but no anastomosis. The nervous system of vertebrates Bethe believes to be somewhat differently constituted. On the surface of the nerve cells and dendrites occurs a network, first observed by Golgi, and termed the Golgi net by Bethe. Bethe regards this superficial Golgi net as being nervous in nature and homologous with the "fibrillengitter" of the invertebrate nerve cord. Into it pass the fibrils from the sensory neurones, and from it pass fibrils which enter the nerve cells and become its neurofibrils. There is thus a neurofibril continuity via a superficial nervous Golgi net.

Cajal regards the Golgi net as a mere coagulation artefact. In a recent paper Marui (19) believes it to be nervous in nature, but an examination of his figures reveals a most obvious coagulated "foam." I have recently observed the Golgi net on the surface of nerve cells from the spinal cord; I find that it shows a special tendency to occur in regions where the intercellular matrix has undergone "frothing," and no evidence whatever is available to show that it is anything but a very obvious artefact.

Held (14) has described a "pericellular nerve terminal net" on the surface of the nerve cells, quite distinct from the Golgi net. It has apparently arisen from the fusion of neurones. The "axospongium" of the nerve terminals is described as "loosening," and having "neurosomes" densely embedded in it. These end-buds he describes as having a definite connection with the cell body. With the use of the reduced silver method, Held in 1905 maintained the continuity of the fibrils of the axons and the neurofibrils (15). Holmgren confirms this (16). Bielschowsky (8) describes a direct continuity between the end-knobs and the protoplasm of the Purkinje cells of the cerebellum, and attributes Cajal's discontinuity to an incomplete impregnation of the end of the fibre.

More recently Bartelmez (5) describes a contact relationship of fibres from the vestibular nerve with the giant Mauthner cell of fishes and tailed amphibians, while Marui (19), using the same material, finds that the neurofibrils from the fibres of the vestibular nerve actually penetrate the body of Mauthner's cell, thus essentially confirming Held's conclusion on this point. In a recent paper F. M. Ballantyne (4) finds that the fine fibres which traverse the grey matter of the spinal cord in *Lepidosiren* penetrate the nerve cells, without the formation of end-buds, and are continued into the cell as its neurofibrils.

In my own studies on the nerve cells of young mammals (rabbit, guinea-pig, cat) I find (23, 24) that the fine fibres (collaterals) that are to be observed in thousands in the grey matter of well-impregnated spinal cord sections converge upon the dendrites of the nerve cells, penetrate them, and are continued within these as the neurofibrils of the cell. In the centre of the cell these fibrils appear to anastomose, and this is apparently the seat of the integration. Fusion of neurofibrils in the dendrites, as described by Kato (17) and others, is probably an artefact, due to the fibres sticking together during the killing of the protoplasm. In these studies no indication of ramifying synapses embracing the nerve cells, or spread out among its dendrites, was observed. It was pointed out that most of the evidence obtained by Cajal for the existence of such structures was invalid; and while it was not possible definitely to exclude the existence of such synapses, yet the predominant type of neurone junction was one of direct continuity; not, indeed, through a superficial Golgi net as described by Bethe, but by the direct entrance of the fibrils into the dendrites of the cells. Occasionally a fibril penetrates the body of the nerve cell directly, but this appears to be quite an exceptional condition, and, so far as I could see, only the dendrites are usually involved.

The Neurone Junctions in Sympathetic Ganglia and in the Ganglia of Auerbach's Plexus.

When we attempt to extend these observations to the sympathetic system special difficulties are encountered. The reduced silver method, which gives such excellent and consistent results with the spinal cord, is far less satisfactory when applied to the sympathetic ganglia. The fixation is often bad, and neurofibril impregnation often does not take place. Under these circumstances one finds in practice that it is best to make a large number of preparations, and discard all those which are inferior. What underlies this irregularity in the staining is not known; possibly it depends upon the density of the fatty tissue that often surrounds the ganglia. The second difficulty that is encountered is that the collaterals from the neurones that enter the ganglion do not run approximately in one plane, as they usually do in the cord, but on the contrary run a most irregular course through the ganglion. While, therefore, in the cord we are able to trace bundles of collaterals, from the white matter almost to their termination in the grey, bundles of collaterals are so ill-defined in the sympathetic ganglia that this is not possible.

The observations recorded here have been made with the use of Cajal's reduced silver method, and the methylene blue technique. For staining the thoracic ganglia I have used Ehrlich's method of injecting a weak solution of methylene blue through the dorsal aorta of a freshly killed animal; for the ganglia of Auerbach's plexus Dogiel's immersion method gives excellent results. With the silver method it is advisable always to use young animals, as these seem to give a much better neurofibril impregnation. For material I have used thoracic ganglia from newly-born rabbits, guinea-pigs, and kittens, unborn calf, and ganglia from fully grown frogs, lumbar and coeliac ganglia from adult men, Auerbach's plexus in young rabbits and guinea-pigs, and in fully grown frogs.

Since the structure of the peripheral intestinal plexuses, as Dogiel has shown (11, 12), is closely similar to that of the sympathetic ganglia, I have thought it advisable to supplement the study of the latter by a careful examination of Auerbach's plexus. This is facilitated by the fact that these widely dispersed plexuses obviously offer ideal conditions for studying the structure of neurone relationship. A description of the human sympathetic ganglia will be deferred till the end of the paper, since these ganglia, at first sight, differ in some important respects from the sympathetic ganglia of animals.

Spinal medullated nerves enter the sympathetic ganglia via the white rami; within the ganglia they appear either to terminate in a large arborization, or they may pass right through the ganglia, usually giving off one or more collaterals to it, and finally terminate in some other ganglion higher or lower in the chain. These arborizations have been observed by Cajal (9), Kölliker, van Gehuchten, and others. In drawings which Cajal has given, they are shown ramifying over a large part of the ganglion. Thus, in figure 550 of Cajal's book, axis cylinder arborizations are shown traversing a region of the ganglion four or five times that which would be occupied by a single nerve cell and all its dendrites. In figure 554, taken from a young chick, the collateral arborizations occupy about the same area as that occupied by a single nerve cell and its dendrites. What is the relation of these collaterals to the sympathetic cells? It has been pointed out by Cajal that a terminal arborization comes into contact with a large number of sympathetic cells, but the actual relation of the arborization to the nerve cells he did not observe.

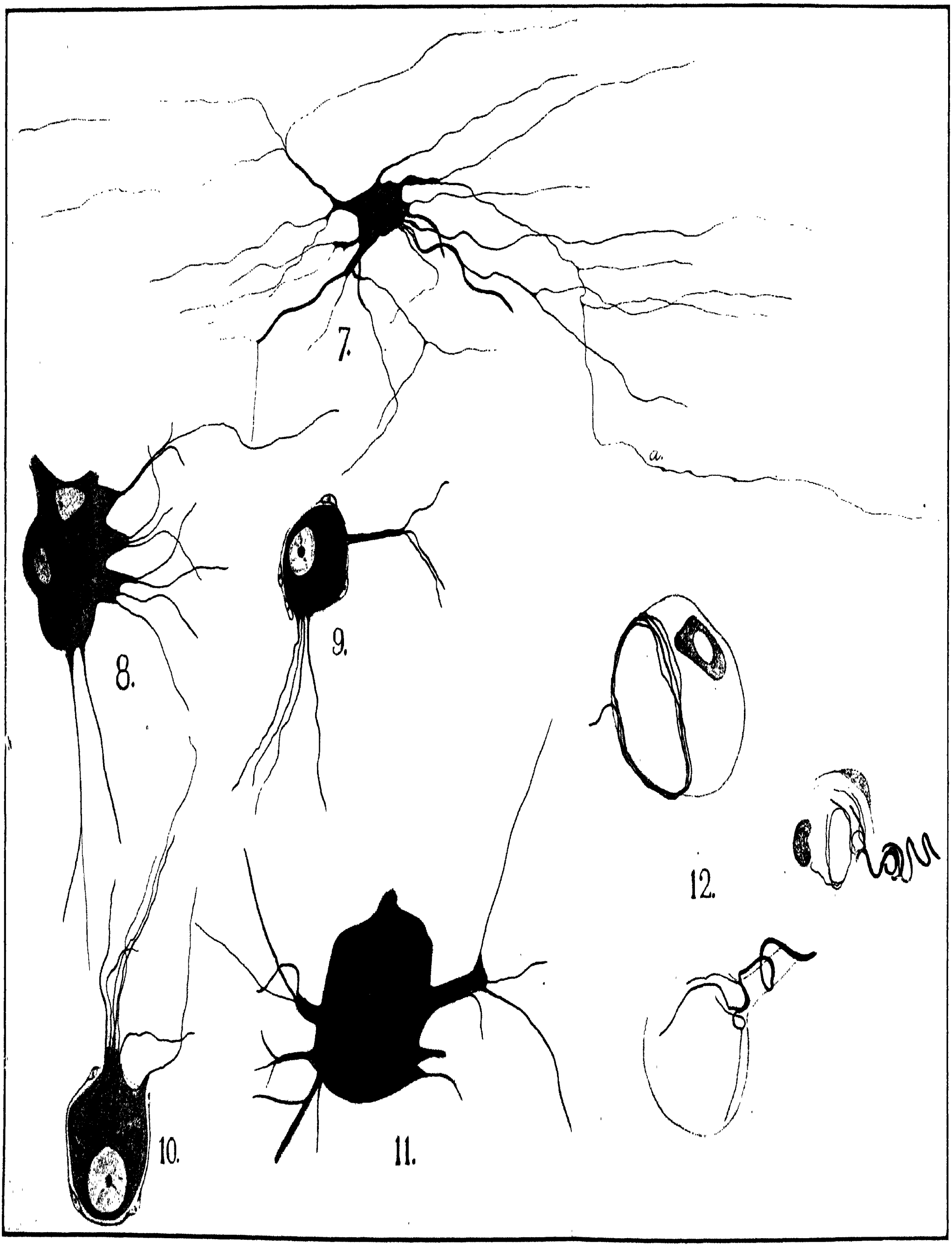
In the cells of the spinal cord, as I have previously found, the predominant type of neurone junction is in the form of collaterals penetrating the cell dendrites. Although Cajal has described ramifying terminations of nerve fibres ending as pericellular expansions upon the surface of posterior horn cells or their dendrites, I have not been able to observe these. In the sympathetic cells, on the contrary, their presence may be definitely affirmed. They were first observed in the frog's sympathetic by Beale in 1863 (6), and by Arnold (2), long before the introduction of special staining methods. In this situation they have also been described by Aronson and by Retzius in the rabbit; Warfwinge (26) has observed them in the frog; they have also been found by Cajal and by Kölliker, the latter observer finding sometimes that more than one fibre may be involved in this pericellular termination. Excellent illustrations of such terminations occurring in sympathetic ganglia of fish, amphibia, reptiles, birds, and mammals, have also been given by Huber (18). Dogiel (11) has likewise observed these pericellular terminations on the cells of Auerbach's plexus. In this case they arise from fine medullated fibres, and are therefore probably derived from the vagus nerve, and not from the sympathetic.

Using the reduced silver method, I have had no difficulty in rendering these pericellular terminations visible in the frog (see figure 12). In this animal the fibre, on approaching the cell, winds itself spirally round its axon, as Beale and Arnold observed over sixty years ago, and usually ramifies upon its surface. Occasionally the ramification does not seem to occur, and the fibre winds itself round and round the cell (figure 12). In figure 15 is shown a nerve cell from Auerbach's plexus, with two pericellular endings upon its surface.

It must be admitted that we have here a true pericellular termination of a kind whose existence in the spinal cord cannot yet be accepted. Lest it be suspected that this in itself contradicts my previous observation on the spinal cord, it may be pointed out that the frog's sympathetic is in this respect peculiar. The ganglion cells in the frog are devoid of dendrites; consequently a neurone junction via cell dendrites cannot occur. I find, therefore, in agreement with Ranson and Billingsley (21) that this is the only type of neurone junction occurring in the sympathetic ganglia of the frog. Although Huber (18) has found similar terminations in mammals, it will be pointed out below that in this class conditions are more complex, and a definite penetration of collaterals into the cell dendrites constitutes the predominant type of neurone junction here, a type of junction, in short, of the same kind as I find in the spinal cord. This conclusion is supported by the observations of Ranson and Billingsley, who find a highly developed intercellular plexus between the ganglion cells of mammals, a plexus which degenerates upon section of the preganglionic fibres. What the relation of this enormous number of fibrils to the ganglion cells is, they do not directly observe, but believe that there can be little doubt that synaptic relations exist between them and the dendritic ramifications. It will be shown below that this "synaptic relationship" is of the same type as that occurring in the spinal cord, namely, that the collaterals penetrate the dendrites and become the neurofibrils of the cell.

Although, it is true, these preparations from the frog clearly reveal a definite pericellular termination on the surface of the sympathetic cell, they do not enable us to say whether it is a case of contact or of actual fusion of the two neurones. In surface view the terminal expansion certainly seems to lie upon the outside of the cell, and Ehrlich many years ago observed the fine terminal fibre, stained with methylene blue upon the surface of the cell, and not apparently entering its substance. These observations, however, throw but little light upon the question; perfect preparations of the endings of motor nerves on "striated" muscle fibres, when seen in surface view, seem to reveal the end-plate as merely lying upon the surface of the fibre; nevertheless, it has been found, especially by Boeke, that these motor nerve fibres have penetrated below the fibre, and have entered its substance. The case of motor endings on muscle, indeed, is of particular interest when we recall the discussion which has prevailed in the past concerning the hypolemmal or epilemmal nature of these endings. The case of the motor nerve endings upon muscle fibres illustrates the futility of the argument, often advanced, that a neurone discontinuity must exist, on the ground that a pericellular ending may stain sharply with methylene blue or with silver-chromate, while the cell upon which it ends remains unstained; for in the case of the motor endings precisely the same may be observed. If a frog's sartorius muscle be immersed in a little methylene blue, and kept under observation, it is found that many of the nerve fibres and their terminal plates stain a deep blue. while the muscle fibre remains unstained; but one also observes many muscle fibres which stain strongly, but in which the motor end-plate remains unstained; yet we would not be justified in concluding that therefore the motor nerve merely lies in contact with the muscle fibre. Similarly, the teaching of Löwit, that the motor endings on plain muscle fibres are intraprotoplasmic (hypolemmal) in position has been contradicted by Cajal, Retzius, Arnstein, and others, nevertheless, it has been clearly shown by Boeke, and by myself (25), that Löwit's original contention is correct.

We conclude, consequently, that while in the spinal cord pericellular terminations have not yet been satisfactorily demonstrated, in the sympathetic cells they certainly occur; at the same time the observations are insufficiently precise to enable us to determine whether the endings have penetrated the cell or whether they merely lie upon its surface. Analogy with motor endings on muscle certainly suggests that the former is the more probable.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But we cannot necessarily conclude from this that the sympathetic cells are fundamentally different from the cells of the spinal cord. When preparations of mammalian sympathetic cells, made by the reduced silver method, are examined, it is found that the neurofibrils which form a very open network within the cells emerge from these, and traverse long distances of the ganglia as naked extracellular neurofibrils. This is clearly shown in figures 1, 8, 9, 10.

Numerous figures of the neurofibril organization of the sympathetic cells have been given by Cajal, by Warfwinge, and by others. These drawings reveal an extraordinarily dense meshwork of the very finest fibrils, pervading the whole nerve cell and its dendrites. I have obtained preparations showing the same condition, and have observed it also in the posterior root ganglia. These neurofibrils have a very different appearance from the neurofibrils which the same method can reveal with such astonishing clearness in the cells of the spinal cord. I strongly suspect that we are concerned here with an artificial product of the fixation of the cell protoplasm, and that the neurofibrils are in no way the same as the neurofibrils revealed in the spinal cord cells. They appear to be one of the numerous artefacts obtained with the use of an as yet very imperfect technique. I find, on the contrary, here and there among the many cells examined well-fixed cells showing neurofibrils of precisely the same kind as are found in the spinal cord cells. Such a cell is shown in figure 1. Lest it be urged that here, too, we are victims of an artefact, it may be remarked that the main evidence in favour of regarding these neurofibrils as definite cell entities is that they can be traced as independent fibrils along the dendrites, and then emerge and traverse the ganglia as naked extracellular neurofibrils.

Now when these naked fibrils are traced outwards from the cells we find that they either enter the bundles of other similar or thicker fibrils—in which case they cannot any longer be individually followed—or, as often happens, after tracing them for considerable distances, they emerge from the plane of the section. Frequently, and especially is this the case at the surface of the ganglia, the cells are devoid of dendrites, and the neurofibrils emerge directly from the cell body and traverse the ganglia as naked neurofibrils (figure 9).

Now if thick sections of sympathetic ganglia, prepared by the Ehrlich method, or the reduced silver process, be examined, we can observe many cases of axons branching considerably in the substance of the ganglion, probably mainly arborizing collaterals from medullated spinal nerves (see figure 2). The fine fibrils which thus arise can be followed often for great distances. They are usually of extraordinary delicacy, and much finer than the spiral fibre which forms pericellular endings around the ganglion cells. I have examined many hundreds of these fine collaterals, almost always with the same result. Like the naked neurofibrils that emerge from the nerve cells, and to which they are similar in appearance, they can be traced either into a bundle of fibrils, within which they cannot any longer be followed, or they leave the plane of the section. The path of the fibres within the ganglion is, however, so irregular that we are even less likely to trace such a collateral to its termination than we are in the case of the spinal cord, where, indeed, it is difficult enough. During a prolonged examination of many hundreds of such collaterals, extending over a period of several weeks, I have succeeded in finding only a few cases where such a collateral could be traced to its ending. In these cases, some of which are shown in figures 3, 4, and 5, the collateral was a very short one, and not so irregular in its path as to emerge from the plane of the section before reaching its destination.

A careful study of the minute structure of the sympathetic ganglion seems to show, therefore, that in the ganglion we have two types of neurone junction; there is a direct neurone continuity similar to that found in the cord, in which fine branches from the arborizing collaterals travel an irregular path in the substance of the ganglion, and penetrate the dendrites of the cells; or if, as often happens in sympathetic ganglion cells, dendrites are few in number, directly enter the cell body (this is the method by which the intercellular plexus of Ranson and Billingsley ends); and secondly, there occur pericellular endings enveloping the cells, and probably penetrating its substance, though this has not yet actually been observed. The latter occur in mammals (Huber), and constitute the sole type of neurone junction in the ganglia of the frog.

Lest it be urged that there is little real evidence in favour of the former type of junction, it may be pointed out that though this has been but rarely observed *directly*, we are forced to this conclusion on certain grounds. Firstly, there occurs seldom more than one pericellular ending upon the "surface" of the nerve cell. A convergence of neurones upon a final common path would not therefore seem to occur, and integration would not be possible.[1] Secondly, in well-fixed reduced silver preparations we find that the fine branches of the collateral arborizations are much too thin to be identical with the fibre which forms the pericellular ending. There must occur, therefore, a neurone junction quite apart from the thick pericellular ending. The fact that this second type of neurone junction, which I regard as the predominant one for the sympathetic ganglia of the mammal, is so extraordinarily difficult, if not almost impossible to demonstrate *directly*, cannot be taken as an argument against its existence; for such a junction could only be directly revealed if the fibrils ran a very regular course within the ganglia, and this they certainly never do. This much is definite: that for almost every axon collateral that we observe under the microscope no real ending can be found.

In methylene blue preparations of sympathetic ganglia, in cases where only a few cells have been stained, it is possible to observe fine fibrils "leaving" the nerve cells (figure 7). Of these one is the axon, and is usually recognized by the collaterals that arise from it. What are the other very fine fibrils that seem to grow out from the cell? If we trace them away from the cell they either emerge from the plane of the section or gradually fade out. This does not signify a true ending of the "dendrites," for their ending is never a sharp one, but is due to a gradual failure of the stain to impregnate them further. These "dendrites" are indistinguishable in appearance from fine nerve fibres, and they cannot be shown to have any relation to other nerve cells. Nevertheless, to dismiss them with the name "dendrite" does not seem to throw much light upon their real nature; they are quite indistinguishable from fine nerve fibres in appearance, but they cannot be shown to have any relation with other nerve cells. Furthermore, there does not seem any reason to homologize them with the Type 11 cells found by Dogiel in the myenteric plexus, and believed by that investigator to be sensory. In Auerbach's plexus these Type 11 cells are very prominent, and their several long dendrites are stated (though Cajal could not confirm this) to terminate upon the mucosa of the intestine. This would readily establish their sensory nature, but it does not seem possible to extend this idea to the cells of the sympathetic chain. At any rate, there is as yet no evidence to show that the numerous long, fine "dendrites" of these nerve cells emerge from the chain and end as sensory structures upon some viscus. Their nature seems a mystery, unless we put the obvious interpretation upon them, and regard them as fine neurones that have grown into the sympathetic cell. A comparison of figure 8 with figure 7 will make this more convincing.

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[1] In the case of the frog it must be admitted, however, there is no evidence that such integration occurs, for, as a rule, only one pericellular ending is seen on the surface of a single nerve cell.

The conclusion above reached, namely, that there are two distinct types of neurone junction in the sympathetic ganglia derives strong support from the observations of Dogiel upon the structure of Auerbach's plexus, and the nerve plexus in the gall bladder (11, 12). Two types of neurone junction certainly occur here. There are pericellular arborizations, enveloping the body of the cells, derived from fine medullated nerves, and therefore belonging apparently to the vagus nerve. But most of the fibres of the plexus are non-medullated nerves. These traverse the ganglia, giving off numerous collaterals, and eventually terminating in a distant ganglion. These collaterals[2] are described by Dogiel as breaking up into the very finest threads, which ramify amongst the short dendrites of the motor cells of the ganglion (Dogiel type 1 cells). The precise relation of these very fine fibrils to the motor cells Dogiel could not determine; nevertheless, he believes that they merely come into contact with the dendrites of these cells.

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[2] According to Dogiel the unmedullated fibres which traverse the plexus and give off collaterals in the various ganglia are in part sympathetic in origin, and in part arise from the motor cells of the plexus. He believes that the sympathetic fibres terminate on the cells of the plexus, and that such neurone relationship is not therefore confined to the vagal nerve fibres. On the other hand, Gaskell is of the opinion that the nerve cells of Auerbach's plexus are entirely vagal in origin, and that the vagal fibres alone form synapses with these cells. The sympathetic fibres, on the other hand, he believes, run an independent course to the musculature. The nerve cells of the plexus accordingly constitute the post-ganglionic portion of the vagus nerve, and the enteric system conforms, therefore, to the ordinary two-neurone (pre- and post- ganglionic) type, as found in the rest of the autonomic system. In favour of this is quoted the fact that nicotine abolishes the effects of vagal stimulation, leaving the effects of sympathetic stimulation unchanged (See W. H. Gaskell, "The Involuntary Nervous System"). This conclusion encounters the difficulty that the terminations of vagus fibres appear to be but few in number. Langley has noticed this same difficulty (see Langley, Journ. Physiol., vol. lvi, p. xxxix). He points out that while the vagal

The actual relation of these fine fibres to the motor cells is extremely difficult to observe, and this is due to the peculiarities of the methods of staining used. With the silver-chromate method Cajal is able to reveal with great clearness the myriads of fine fibrils that traverse the plexus, while at the same time the method leaves the motor cells of Dogiel unstained. It is obviously impossible under these circumstances to determine the relation of the fibrils to the motor cells.

The methylene blue process is usually equally indefinite. It has been my experience, and Dogiel's figure seem to indicate the same, that in preparations which clearly reveal the nerve cells of the ganglia, these nerve fibres are almost entirely unstained. The opposite also holds; and in preparations in which a thick feltwork of fine fibrils is strongly stained, nerve cells are recognizable only as clear, unstained spaces amidst the mass of fibres. Such preparations are again obviously unsuited for determining the relation of the fibrils to the nerve cells.

Nevertheless, if one examines a large series of ganglia, one occasionally finds cases where one or more nerve cells have been sharply stained, and where at the same time a small number of fibrils are rendered visible. It is these ganglia that throw most light upon the structure of the neurone junctions, and in these definite examples of neurone continuity may be detected. In figure 14 is shown a small motor cell from the myenteric plexus of the frog *(Hyla aurea)*. In a bundle of fine longitudinally running fibres (they have not been shown in the figure), one single fibre has stained sharply; it presents two varicosities, and gives off almost at right angles a very distinct collateral, which communicates directly with a small motor cell.

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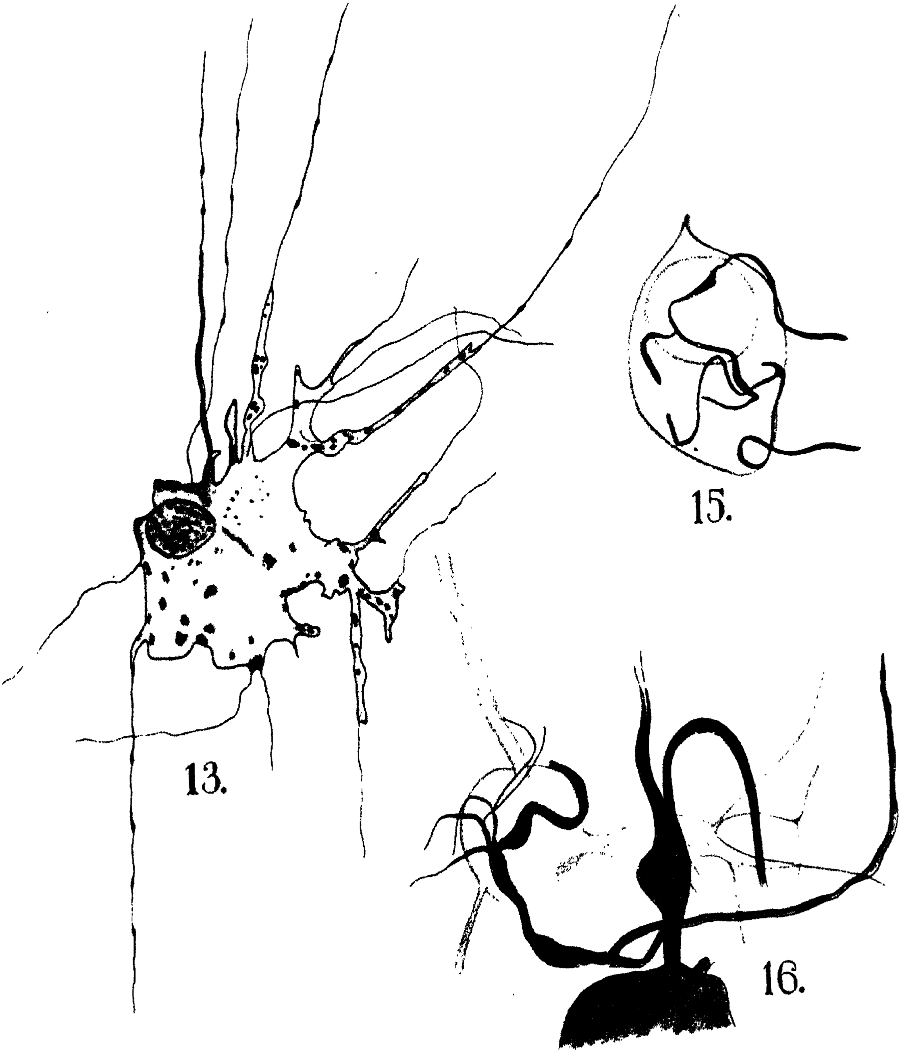
supply to the gut contains relatively few fibres, the cells of the plexus which this system supplies are to be counted probably in millions. He believes that the vagus fibres can terminate upon a relatively small number only of the cells of the plexus, and that the axons from these nerve cells branch extensively, and form junctions with the remaining cells of the plexus. This view is perhaps more in agreement with the findings of Dogiel, and is strongly supported by the studies of Erik Müller (Arch. f. mikr. Anat., 94 (1920), p. 208), on the development of the enteric nervous system in Squalus. On the other hand, it does not conform in plan with Gaskell's two-neurone system.

The view of Langley carries with it the possibility that the sympathetic nerve fibres might end upon the terminal cells of the vagal system, for we cannot now argue that nicotine would necessarily paralyze these cells in the manner in which it paralyzes ordinary post-ganglionic neurones. This might mean that the sympathetic and vagal nerve cells do not run an entirely independent course to the gut musculature, but that they converge upon a final common path; Dogiel's conclusions would then be correct. It does not seem, however, that pharmacological evidence can throw further light upon the question. Whatever the truth may be, it is fairly clear that the neurone terminations on the cells of Auerbach's plexus must have a double origin, one vagal (extrinsic), the other intrinsic; but whether the sympathetic fibres also form junctions with this system cannot yet be stated.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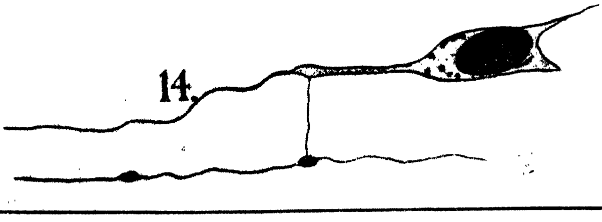

14.

I find, moreover, that if the individual cells of the plexus are examined, fine fibrils may be seen apparently emerging from the dendrites. In the ganglion cells of the frog which I have examined, the great majority of the cells show such delicate short fibrils, apparently growing out of the dendrites. Sometimes they can be traced a considerable distance from the cell; at other times this is impossible, so faint and ill-defined do they become as we follow them away from the dendrites. What are these fine fibrils? That they are in direct connection with the dendrites cannot be doubted; figure 13 shows this with the greatest clearness. They are quite indistinguishable from nerve fibrils, and present the same fine varicosities as do these. Just as in figure 15 we see two pericellular expansions ending upon the body of a nerve cell, and at the same time showing no connection with the medullated fibre with which we know them to be connected, so it seems most obvious to conclude that these fine fibrils that enter the dendrites of the ganglion cells are the terminations of otherwise unstained non-medullated fibrils whose mode of ending Dogiel could not determine. There is, however, an objection that may be raised against this conclusion that we must carefully consider.

In Auerbach's plexus there are, as Dogiel has found, two types of cell (a third type which he has occasionally found does not concern us here). One of these, the type 1 (motor) cell, is characterized by the presence of short, slightly branched dendrites, and a single axon; the other, type 2 cell, is regarded as sensory. It is characterized by the presence of enormously long dendrites, indistinguishable from the axon in appearance, and terminating apparently in the epithelium of the intestine. The main point, then, by which we distinguish the two types of cell is that in the type 2 cell the short branching dendrites that characterize the type 1 cell are absent, so that the cell body has a relatively smooth contour, with a small number of extremely long, fine, varicose dendrites growing out from it. Nevertheless, Dogiel occasionally figures a cell, stated to belong to type 2, in which short dendrites occur, and from the ends of which the fine varicose fibres grow out.

Apart from such a fortunate case as that which I have shown in figure 14, where a fine branching fibre becomes continuous with a motor cell, the only other evidence which it is possible to get for a direct neurone continuity, is to observe fine fibrils, in the manner described above, apparently growing out from the nerve cells. Since in preparations where the nerve cells are clearly stained nerve fibres do not usually stain, it will be obviously impossible to trace these fine fibrils any great distance. Consequently, whenever such a cell is observed it will naturally be designated a type 2 cell, whose dendrites have incompletely stained, rather than a type 1 motor cell, into which fine fibrils (collaterals) from other cells have penetrated. Under these circumstances it is obviously impossible to obtain evidence for the growth of fibres into the dendrites of type 1 cells, since every time we find such a case we unhesitatingly call it a type 2 cell. But it is very doubtful whether this procedure is justified. The type 1 cells are characterized by the presence of numerous short, branched dendrites, which are not typical of the type 2 cells. When we find cases of such cells, with fine fibrils apparently growing out of the dendrites, we seem much more justified in considering them as genuine cases of collaterals penetrating the type 1 nerve cells, than merely as abnormal cases of type 2 cells, with incompletely stained dendrites. In figure 13 is shown such a cell, into which at least fourteen fine fibrils seem to have penetrated. We are clearly not justified in calling this a type 2 cell, when in actual appearance it is indistinguishable from a type 1 cell (see, for example, figure 4b of Dogiel's paper).

The general conclusion which we draw from these observations is therefore as follows: *In the cells of the ganglia of the sympathetic trunk, and of Auerbach's plexus, two distinct types of neurone junction occur; fine collaterals penetrate the dendrites or the body of the nerve cells, and (this has been seen in the sympathetic ganglia only) become the neurofibrils of the nerve cells, undergoing anastomosis with the neurofibrils that have arisen in a similar way from other fine collaterals which have penetrated the cell. This anastomosing network is the seat of the integration. In addition to this type of neurone junction, which is the predominant one in the mammal, but does not occur in the sympathetic ganglia of the frog, there occur pericellular end-ramifications of nerves (seldom more than one on a cell), but it is not yet possible to say whether these end upon the surfaces of the cell or penetrate into its substance.*

Observations on Human Sympathetic Ganglia.

Special attention has been paid here to the structure of the neurone junctions in human sympathetic ganglia, since it is in this material that Cajal has made some of his most striking observations on neurone discontinuity at the synapse. Cajal's observations were made on the superior cervical ganglion.

In this ganglion Cajal distinguishes three main types of cell: (*a*) Cells with very long dendrites, (*b*) Cells with very short dendrites ending within the capsule, and (*c*) Cells having both types of dendrites. The last do not concern us here, since they merely combine the characters of the first two.

Concerning the first group, Cajal has observed that there are usually a very small number of neurones enveloping the body of the cells, whilst fibres may also be observed wound spirally round the dendrites. The spirally wound fibres, and the fibres which are wound round the body of the cells, constitute, he believes, the actual terminations of fibres upon this type of cell, i.e., the junction is believed to be one of contact and not of continuity. Of the dendrites it is stated that they divide occasionally, and eventually pass among bundles of fibres, often very far from their cell of origin, and eventually become lost. It is inferred, of course, that they eventually end blindly.

Concerning the second group, Cajal finds that the dendrites may be of two different kinds: thick, blunt dendrites, and very fine, delicate dendrites. The latter may break up into a bundle of very fine fibrils, which end generally close to their point of origin, just within the capsule that surrounds the cell. These cells are well supplied, at times extraordinarily so, with very delicate fibres which penetrate the capsule from without and ramify extensively among the thick and thin dendrites; again, the neurone junction is therefore believed to be one of approximation of neurones, rather than one of continuity. Cajal admits at the same time that the mass of fine fibres is so complex that it is impossible to unravel it and trace the fibrils individually.

My own observations have been made on human lumbar and coeliac ganglia, prepared by the reduced silver method. The former was from a man aged thirty years, the latter from an old man of seventy. Despite the fact that the ganglia were obtained more than eight hours after death, they gave excellent preparations, except that no indication of the internal neurofibril organization of the cells could be detected.

The three types of cell described by Cajal are readily detected, but the type with very short subcapsular dendrites is uncommon. In these ganglia I have not observed cells showing such extraordinary complexity as some of those figured by Cajal from the superior cervical ganglion. This really constitutes a considerable advantage, since in these cells of lesser complexity we have a much greater opportunity for following the individual fibres to their destination than in those complicated examples figured by Cajal, which, he himself states, he was unable to unravel.

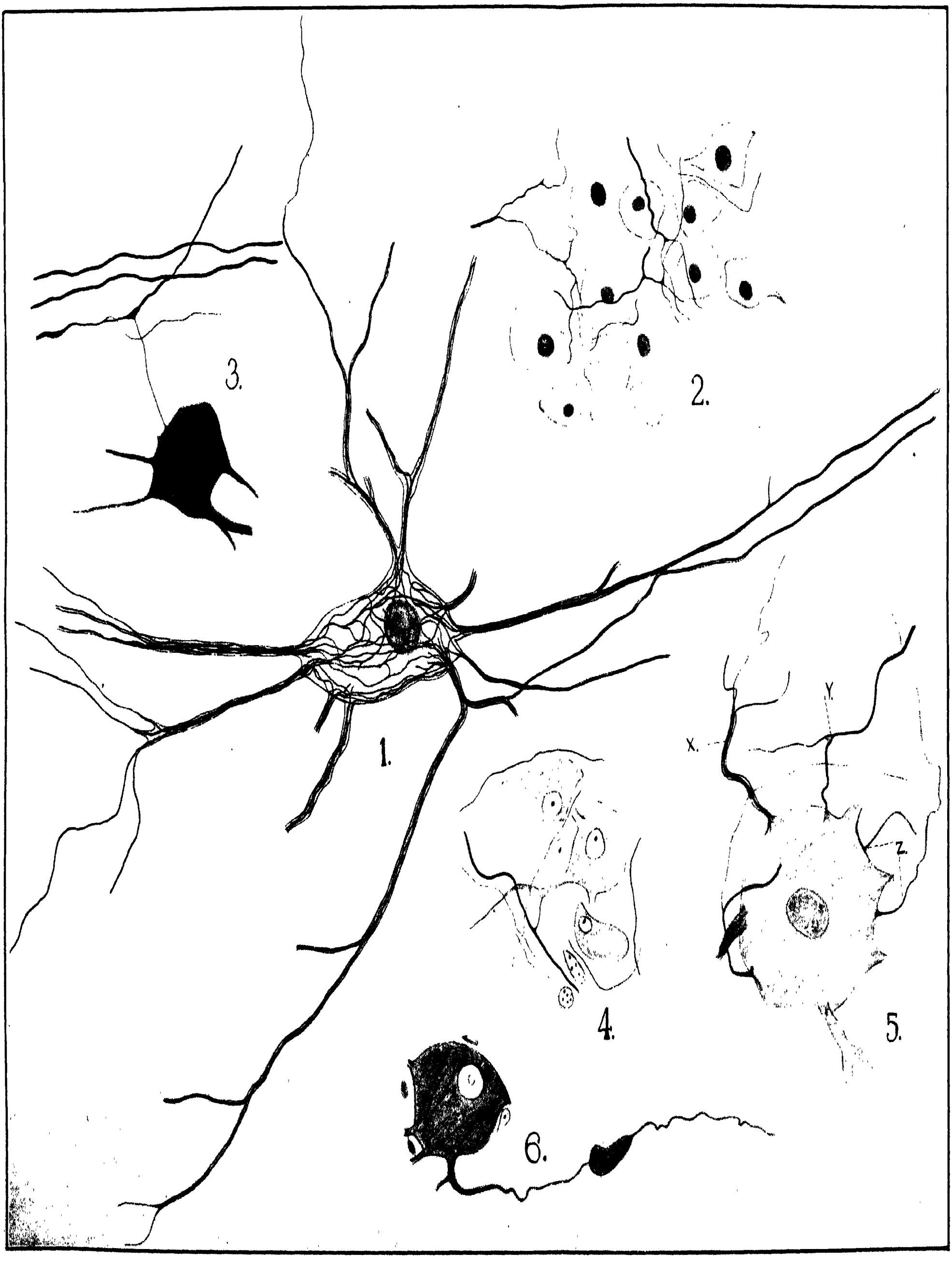
I shall first describe the cells with dendrites which pass outside the cell capsule. In the lumbar and coeliac ganglia these constitute by far the majority of the cells. Sometimes the dendrites are quite delicate; at other times they are thick and massive, branch extensively, and become finer as we trace them a long way from the cell. Cajal has repeatedly stated that these various dendrites *terminate* sooner or later; my own observations show that this conclusion needs modifying. In the case of the fine dendrites, I find, as described by Cajal, that they usually break up into a few fine fibrils; but these fibrils, into which the dendrite has split, appear to terminate *because they pass out of the plane of the section.* A case of this is shown at X in figure 5. The short dendrite has split up into three fine fibres, but these only terminate because they emerge from the plane of the section; it is not, of course, possible to represent this on paper. When the large, thick dendrites are followed away from the cell, it is found that they undergo the same fate, but on a larger scale. They give off side-branches, which again branch in turn. These branches, which may be thick or thin (and eventually the dendrite itself also), after a longer or shorter distance, either become lost amongst other groups of fibres, or pass out of the plane of the section.

In such cases again it is impossible to detect any real termination of the "dendrite." When we observe the fine branches into which the dendrites have apparently divided, we observe in these a close resemblance to branches of axons. Those who maintain the theory of ramifying synapses ending upon nerve cells and their dendrites, will see in these fibres that have been given off from the dendrite merely an extensively branching dendrite system, and it is certainly difficult to disprove this assertion. There are, however, two objections which may be raised against it. Firstly, in the ganglia which I have examined most of the cells with long dendrites are quite devoid of fibres wound spirally round the dendrites, or terminating upon the body of the cell. It would be useless to object that they have merely not been rendered visible by the stain, for the staining was extraordinarily good. In this case we would have to conclude that these cells had practically no neurones ending upon them, which would be absurd. It seems much more probable that the fine fibres that appear to grow out from the dendrites are in reality branches of axons that have grown *into* the dendrites, in exactly the same manner as I have described in the cells of the spinal cord. It is possible to obtain more direct proof of this.

As I have pointed out in a previous paper (22), there are two means whereby we can recognize axon arborizations in sections of silver-nitrate material. The first is to trace these branches to the nerve cells from which they have originated, a method which is almost never applicable in practice. The second is to recognize the axon by the fact that it undergoes branching, there being usually a small triangular piece of protoplasm at the point where the branch is given off. The branch, even if it subsequently recurves, is always given off in the direction in which the axon is running, and it is in the more or less acute angle which this branch forms with the rest of the axon that the small triangular piece of protoplasm is situated.

The proof of this statement lies in the fact that if we observe a single well-defined axon in our preparations, its branches are all found to be given off in the one (forward) direction, and the triangular piece of protoplasm is always situated in the more acute angle which the branch forms with the axon.

While, therefore, the former method of detecting axons is of little value in this work, the latter can sometimes be usefully employed. Usually, it must be admitted, the branches which thus arise from the axons cannot be traced very far, for they soon leave the plane of the section, in the same way as do the branches which appear to grow out from the dendrites. But if very large numbers of such branches are patiently examined, sooner or later one is found which reaches its destination before it leaves the plane of the section. An excellent illustration of such a case is shown in figure 5 at Y, where the branch that arises from a neurone travels only a very small distance, and then grows into a large nerve cell. The neurone junction is obviously of a similar nature to that shown in figures 3 and 4, taken from the stellate ganglion of an unborn calf.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Now in figure 5 are shown a number of other fine fibres (dendrites we might call them), which after travelling a longer or shorter distance from the nerve cell pass out of the plane of the section. They have precisely the same appearance as the branching collateral Y. Doubtless, had the section been cut slightly differently, so as not to reveal the true nature of this latter fibre, it too would have been called a dendrite. It appears, therefore, that many of the very fine "dendrites" that seem to grow out from the cell are not dendrites, but rather branches from axons which have grown into the cell. At X, on the other hand, is shown a dendrite which breaks up into three fine branches, all of which emerge from the plane of the section. It is difficult to avoid the conclusion that these also are fine axon collaterals that have grown into the cell.

When we examine the fine branches which "grow out" from the large dendrites of the nerve cells, precisely the same observations may be made. Usually they leave the plane of the section, or become lost among a bundle of other similar fibres. But occasionally one is able to observe that such a branch, which seems to have grown out from the dendrite, is in reality the collateral from an axon, and therefore has evidently grown *into* the dendrite. The case is perhaps made most clear by reference to figure 11, which shows a thick but very short dendrite, obviously an outgrowth from the cell. From the ends of this dendrite there appear to grow out four fine fibres; they pass eventually out of the plane of the section, and we must look upon them, in reality, not as outgrowths from the dendrite, but as axon collaterals that have grown into the dendrite.

Similar remarks apply to the second type of cell seen by Cajal, those with dendrites ending within the cell capsule. These dendrites may be thick or very thin and delicate. My observations have been made only on the delicate ones. These may or may not break up into smaller branches, but in all cases when these branches are followed away from the cell they never end (as Cajal has maintained) beneath the capsule, but their ending is always a false one. Sometimes it is possible to trace them for but a short distance; at other times they may be traced a considerable distance within the capsule, but their ending is always an unreal one. Similarly, the fine fibres that penetrate from without into the capsule tend to become indistinguishable as individual fibres in the great mass of fibrils to which they give rise, and their terminations, whenever these occur, are always unreal. Cajal has himself admitted that it is practically impossible to follow individual fibres in this complex structure; how under such circumstances could a neurone discontinuity justly be maintained?

Reference must be made here to a particular type of dendrite relationship to which Cajal seems to attribute a physiological significance. In many sympathetic cells numerous small dendrites are given off from one pole only of the cell, and frequently two or three such cells may be contained within one capsule, with the dendrites mutually ramifying amongst one another. At other times the long dendrites from two cells may break up into small branches and ramify among one another (for an illustration of this see Cajal's book, vol. ii, figure 56, at C and D). As this has been taken to represent a definite type of neurone connection, it may be pointed out that the figures are entirely misleading. The terminations of the dendrites are never real; if sufficient of them be contained in the section they are found to break up into fine fibres, for which no real termination can be detected. We must interpret such cases, therefore, not as definite neurone junctions; on the contrary, it would seem rather that the dendrites are approximated merely because they receive fibres from a common bundle of axon collaterals.

Physiological significance is also attributed by Cajal to cases of dendrites ramifying around the body of nerve cells. On the other hand, Kölliker and van Gehuchten were inclined to regard this as a mere accidental arrangement. I have drawn such a case in figure 16, in which it is seen that the short dendrites of one nerve cell break up into branches, which surround the body of another such cell. Cajal's statement that such dendrites actually terminate upon such a nerve cell seems to depend again upon his failure to realize that the endings which he observes are never anything but apparent endings. In the case shown in figure 16 the fine branches could be traced deeper and deeper down in the section, till they eventually emerged from it.

In human sympathetic ganglia one frequently observes fibres terminating in large, clear, structureless globules. The globules vary in size, but the largest may be nearly half the size of a ganglion cell. Cajal regards them as the termination of dendrites, and believes that they are pathological in nature, produced by the action of toxins and the senescent condition of the subject. I have been able to obtain some evidence in favour of this suggestion of Cajal. While in the ganglion cells of the thirty-year-old man they were entirely absent, they are found in extraordinary profusion in the ganglion of the old man of seventy. Some of them are quite small, others very large, and may often be observed close against a nerve cell, strongly indenting its walls. I have on several occasions observed large dendrites, otherwise normal in appearance, showing a pronounced swelling in one region. But these swellings are not confined to the dendrites, as Cajal believed, but may, on occasion, also be observed on the axons. A very clear case of this is shown in figure 6. One is very tempted in such preparations to suppose that these globules are the result of a gradual degeneration of the nervous elements of the senescent subject, and that such degeneration is manifested in a progressive breaking down of the neurone communications.

APPENDIX.

Note on Nerve Dendrites.

Some investigators have supposed that the dendrites are concerned in the process of nerve conduction; others maintain that they play no part in this, but that their rôle is a nutritional one.[*] The conclusions above reached seem to throw some light upon their nature. The evidence leads us to conclude that axon collaterals, during embryonic life, grow into the body of the nerve cells, either singly or in bundles; the dendrite will then be that portion of the cell protoplasm which has grown outwards from the cell so as to envelop, or to collect within it, these fibrils that have grown into the cell. In reduced silver preparations, in which the dendrites as well as its neurofibrils have stained, this distinction between the dendrites which have grown out of the cell, and the fibrils that have grown into it, is easily observed, and in such preparations, as well as in most methylene blue preparations, the dendrites are found usually to be relatively short, only slightly branched structures. In the spinal cord they are seldom longer than several times the diameter of the cell from which they arise, though in the sympathetic ganglia they are frequently very much longer. But if the same cells are examined in Golgi preparations, they are found to be of enormous length, and in the case of the spinal cord may ramify over an extensive part of the section of the cord. The discrepancy is due to the fact that the Golgi method does not allow us to distinguish between the dendrites and the axon collaterals that have grown into it.

The above view is in harmony with the fact that those cells, such as the posterior root ganglion cells, upon which axon collaterals do not converge, are devoid of dendrites.

SUMMARY.

1. The investigation is an attempt to extend to the sympathetic system and its peripheral plexuses the observations previously made on the spinal cord, of a direct neurone continuity at the neurone junctions.

2. By the use of the methylene blue and reduced silver methods, using material from the cat, rabbit, guinea-pig, calf, frog, and man, it is observed that (except in the frog) the predominant type of ending is the same as that revealed in the spinal cord cells, i.e., the axon collaterals penetrate either into the body of the nerve cell, or more commonly into its dendrites. They pass as neurofibrils towards the centre of the cell, where they anastomose; this anastomosing network appears to be the seat of the integration.

3. In addition there occurs also a second type of ending, in the form of a pericellular ramification of nerve fibres, closely enveloping the body of the cell. This is the only kind found in the frog's sympathetic trunk. The end ramification lies beneath the cell capsule, but it is not possible to state whether it

penetrates into the protoplasm of the cell, or whether it ends in close contact with the cell surface. It is pointed out that a discontinuity of the staining reaction (as seen in Golgi and methylene blue preparations) constitutes no argument for discontinuity in the neurone junction; while, on the other hand, analogy with the motor ending of skeletal muscle is strong evidence in favour of protoplasmic continuity at the junction.

4. It is pointed out that, though the actual growth of axon collaterals into the dendrites constitutes the predominant type of neurone junction, it is only in rare cases that this may be *directly* observed.

5. Human sympathetic ganglia have been specially investigated, and it is concluded that the many remarkable cases of neurone discontinuity figured by Cajal are due to his failure to recognize the dendrite terminations as artefacts, due to their emergence from the plane of the section.

6. In the ganglia of a senescent, but not of a young human subject, the axons and dendrites may degenerate into large globules. Thus is brought about a gradual disruption of the nerve connections within the senescent ganglion.

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Figure 1. Large nerve cell from ganglion stellatum of kitten. Naked neurofibrils are to be seen "emerging from" various dendrites. In all cases the ending of the neurofibrils, and also of the other dendrites, is a false one, due to its leaving the plane of the section. It is, of course, not possible to represent this in the figure. Note that the neurofibrils run an independent course in the dendrites, but appear to undergo a limited amount of anastomosis within the body of the nerve cell.

(Cajal's reduced silver method.)

Figure 2. Nerve cells from thoracic sympathetic ganglion of kitten, amongst which a number of nerve fibres branch.

(Ehrlich's methylene blue injection method.)

Figure 3. From the thoracic sympathetic ganglion of unborn calf. One of the three nerve fibres is shown giving off a fine collateral, which comes into direct connection with a nerve cell.

(Cajal's reduced silver method.)

Figure 4. The same. One of the large nerve fibres is shown giving off a fine collateral, which fuses with one of the dendrites of the nerve cell. Several other fine fibres and three neuroglia nuclei are indicated.

(Cajal's reduced silver method.)

Figure 5. Sympathetic nerve cell with extracapsular dendrites. Two of the dendrites are thick and bulky, and are shown for only a small part of their length. At Y is shown a collateral from another axon, which gives off a moderately thick branch to the cell. No indication of discontinuity at the junction is observable. Two other similar fibres are shown at Z, apparently growing out from the cell (but in reality growing into it). Their relation to the other neurones cannot be demonstrated, since they emerge from the plane of the section. At X is shown a dendrite, into which three axon collaterals have penetrated.

(From Human Coeliac Ganglion of man aged 70 years; Cajal's reduced silver method.)

Figure 6. A nerve cell from coeliac ganglion of 70-years-old man. A large degeneration globule is forming on the axon. An indication of the cell capsule may be observed.

(Cajal's reduced silver method.)

Figure 7. Sympathetic cell from thoracic ganglion of kitten, prepared by Ehrlich's methylene blue injection technique. The axon, which gives off two collaterals, is indicated by a. Of the many fine processes which "grow out" from the cell, all have an unnatural ending, i.e., they may either emerge from the plane of the section, or, as frequently happens, the staining gradually fails. It is argued that these fine fibrils are really axon collaterals that have grown into the cell.

Figure 8. Sympathetic cell from thoracic ganglion of kitten, prepared by Cajal's reduced silver technique. Intracellular neurofibrils are impregnated, though far less satisfactorily than in spinal cord cells. The neurofibrils are observed to emerge from the cell and travel as naked neurofibrils in the ganglion. This cell is evidently of a similar kind to that depicted in figure 7. The distinction between extracellular fibrils and dendrites is much more obvious than in figure 7, and the figure illustrates with greater force the thesis maintained in this paper, that the fine fibrils are not dendritic *outgrowths* from the cells, but axon collaterals that have *entered the cell.*

Figures 9 and 10. The same. The cell capsule is clearly seen in these two cells.

Figure 11. Cell from coeliac ganglion of old man. The distinction between dendrites and the fibrils that have grown into them is obvious.

(Cajal's reduced silver method.)

Figure 12. Three nerve cells from sympathetic ganglion of frog, with pericellular nerve termination. In two of the cells the spiral fibre breaks up into finer fibrils on the surface of the cell. In the other a fibre is wound round and round the nerve cell.

(Cajal's reduced silver method.)

Figure 13. A ''motor'' cell (Dogiel type 1 cell) from Auerbach's plexus. It is maintained that the fine fibrils that appear to grow out from the dendrites are axon collaterals that have grown into the cell. (Duodenum of rabbit; methylene blue.)

Figure 14. A ''motor'' cell from Auerbach's plexus, receiving a fine collateral from a non-medullated nerve fibre. (Frog [*Hyla aurea*]; methylene blue.)

Figure 15. A cell from Auerbach's plexus, with two pericellular nerve-endings. (Rabbit; methylene blue.)

Figure 16. Two nerve cells from human coeliac ganglion. One of the nerve cells (part of which only is shown), gives off a dendrite whose branches surround the body of the second cell. It is observed, however, that these branches are produced into fine fibrils, and that these fibrils end merely because they have been cut short. It is maintained that this does not constitute a termination of the dendrites upon the cell; on the contrary, the fine fibrils are axon collaterals that have entered the dendrites, and the peculiar disposition of the latter around the body of the nerve cell is purely accidental.

(Cajal's reduced silver method.)

WHETHAM'S LAW AND THE LAW OF MASS ACTION; AND THEIR APPLICATION TO THE COAGULATION OF COLLOIDAL PLATINUM

by

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Whetham (13) has generalized the coagulating effect of ions on colloids carrying the opposite charge in the following words: "The coagulating power of a series of ions of the same sign is proportional to a constant raised to the power representing the valence of each ion." This law at the time of its enunciation received quite startling confirmation from the meagre figures at hand, and hence was widely accepted.

Whetham deduced this generalization by the method of probability, but Robertson (11) points out that the same result is obtained by the application of simple mass action principles, such principles being based on the theory of probability.

It is shown in this paper that Whetham's law, and also the law of mass action when based on principles corresponding to those on which Whetham's law is based, lead to quantitative relations which are not supported by experimental facts; and it is concluded that Whetham's law is theoretically unsound, and also that the fundamental assumptions inherent in this particular application of mass law principles are incorrect.

The Application of the Mass Law to the Coagulation of Colloids by Electrolytes.

It will be convenient for the sake of a clear theoretical presentation to discuss the mass law application first, and to divide such discussion into two parts.

(A). The colloid to be precipitated invariably carries an electric charge, and it is assumed that coagulation occurs when the charge is wholly or partially neutralized by combination with an ion carrying the opposite charge.

Suppose that each colloid particle combines with $6n$ unit charges before it is brought into the coagulable state, and for convenience suppose that it is thereby neutralized. Let the concentration of the colloid to be precipitated be Q, and let the concentrations of the uni-, bi-, and tri-valent ions necessary to produce coagulation in equal times be C_1, C_2, and C_3, in gramme ions per litre. As these concentrations are invariably low, C_1, C_2, and C_3 may also be taken as representing the respective activities.

The reactions for univalent, bivalent, and trivalent salts are then given by the following equations:

$$\left.\begin{array}{ll} (1a). & 6n\ Na^{+} + 1\ \text{colloid particle}^{(6n-)} \longrightarrow \text{Colloid}\ .\ (Na)_{6n} \\ (1b). & 3n\ Ca^{++} + 1\ \text{colloid particle}^{(6n-)} \longrightarrow \text{Colloid}\ .\ (Ca)_{3n} \\ (1c). & 2n\ Al^{+++} + 1\ \text{colloid particle}^{(6n-)} \longrightarrow \text{Colloid}\ .\ (Al)_{2n} \end{array}\right\} (1)$$

Now n can be integral or fractional, and in either case the following holds. Calling the initial velocities of the respective reactions V_1, V_2, V_3, and applying the mass law, one obtains:

$$\begin{array}{ll} (1d). & V_1 = k_1\ .\ Q.\ C_1^{6n} \\ (1e). & V_2 = k_2\ .\ Q.\ C_2^{3n}\ \text{and} \\ (1f). & V_3 = k_3\ .\ Q.\ C_3^{2n} \end{array}$$

where K_1, K_2, and K_3 are the respective velocity constants. Now, it is the practice in coagulation experiments to mix the colloid and electrolyte, to hold for some stated time, say, three minutes, ten minutes, or even twenty-four hours, and then to examine the mixture to see if there be any evidence of partial or total coagulation. The smallest concentration of electrolyte which gives definite evidence of coagulation at the end of this fixed time is taken as "the coagulation concentration." It is evident then that this, the usual method, directly compares the coagulation velocities of the reactions concerned. The necessary relations for equal coagulative velocities in the foregoing equations are given by

$$k_1\ .\ Q\ .\ C_1^{6n} = k_2\ .\ Q\ .\ C_2^{3n} - k_3\ .\ Q\ .\ C_3^{2n},$$
$$\text{or}\ k_1C_1^{6n} = k_2C_2^{3n} = k_3C_3^{2n},$$

which is therefore the relation between the concentrations of uni-, bi, and trivalent ions necessary to coagulate a given colloid in a given time.

No simpler relation follows unless we make the simplifying assumption that the three velocity constants are equal, that is, that the three reactions in equation (1) are not chemically specific, but are purely electrical in nature. It must be stressed that this rather sweeping assumption is essential for the carrying further of the general deduction.

With such an assumption we have at once:

$$(1g).\quad C_1^{6n} = C_2^{3n} = C_3^{2n}.$$

Utilizing these relations,

$$(C_1^{6n} = C_2^{3n}\ \text{and}\ C_1^{6n} = C_2^{2n})$$

and taking the $3n$-th root and the $2n$-th root respectively, we get,

$$(1h).\quad C_2 = C_1^2\ \text{and}\ C_3 = C_1^3.$$

We may then write:

$$C_1 : C_2 : C_3 = C_1 : C_1^2 : C_1^3$$
$$\text{or} \quad 1 : C_1 : C_1^2.$$

Using p for the coagulating power (the reciprocal of the coagulating concentration), it follows that

$$(1k). \quad p_1 : p_2 : p_3 = 1 : \frac{1}{C} : \left(\frac{1}{C_1}\right)^2$$

or in the more usual form:

$$1 : X : X^2.$$

Thus the general mass law deduction leads to the usual form of Whetham's law, but with this important difference, the constant X is not an arbitrary constant, but is given by $\frac{1}{C}$. Further, two interesting relations revealed by this deduction are that $C_2 = C_1^2$ and $C_3 = C_1^3$, or the coagulating concentration of the bivalent ion is the square of the coagulating concentration of the univalent ion, and that of the trivalent ion is the cube of the coagulating concentration of the univalent ion.

An interesting point arises when we consider the case where n in equation (1) above is fractional. For instance, if n equals $\frac{1}{2}$ the equations in (1) become:

(2a). $3Na^{+} + 1$ colloid particle $^{(3-)} \longrightarrow$ Colloid . $(Na)_3$

(2b). $\frac{3}{2}\, Ca^{++} + 1$ colloid particle $^{(3-)} \longrightarrow$ Colloid . $(Ca)_{3/2}$ (2)

(2c). $1Al^{+++} + 1$ colloid particle $^{(3-)} \longrightarrow$ Colloid . (Al)

The foregoing general deduction holds if the equations are left in this form; but equation (2b) is an unusual form for the application of the mass law, and if it be thrown into the more usual form,

$3Ca^{++} + 2$ Colloid particles $^{(3-)} \longrightarrow$ $(\text{Colloid})_2 \,.\, Ca_3$,

the value of the velocity constant k_2 becomes altered accordingly. As the general deduction requires the assumption that the three constants, k_1, k_2, and k_3, are equal, it is evident that one cannot be altered unless the other two are correspondingly altered. This then raises the question as to what are really the true comparable settings (if any) for the three equations. A little consideration shows that the most probable settings are those that represent that the same quantity of colloid is coagulated, for experimental measurements are made when the same approximate quantity of coagulated colloid comes into view (or is completely thrown down). If then one of the above equations is multiplied by some factor, e.g., 2, all three equations must be multiplied by this factor. The three velocity constants then become altered to the same degree, and the equations still represent that the same quantities of colloid are coagulated. Under such conditions the relations due to the foregoing deduction will always theoretically follow.

If, however, different factors are introduced into the three equations, then the foregoing relations between the precipitating powers will no longer hold, but quite arbitrary relations, depending on the factors introduced, will be obtained.

(B). Robertson (11) did not use the above settings for the equations, but assuming that one colloid particle combined with one univalent ion, and two colloid particles with one bivalent ion, etc., he wrote the equations:

$$\left.\begin{array}{ll} (3a). & 1Na^{+} + 1 \text{ colloid particle} \longrightarrow \text{Colloid} \,.\, Na \\ (3b). & 1Ca^{++} + 2 \text{ colloid particles} \longrightarrow (\text{Colloid})_2 \,.\, Ca \\ (3c). & 1Al^{+++} + 3 \text{ colloid particles} \longrightarrow (\text{Colloid})_3 \,.\, Al \end{array}\right\} (3)$$

This is equivalent to writing n equal to 1/6 in the general equation (1), and then multiplying (1b) by 2, and (1c) by 3. The introduction of these different factors into the equation should, according to the foregoing, give rise to a different proportionality between the coagulating powers from the general one deduced:

$$\left[p_1 : p_2 : p_3 = 1 : \frac{1}{C_1} : \left(\frac{1}{C_1}\right)^2\right]$$

This is so; for equating the velocities in the actions represented by equation (3) we have:

$$\text{Velocity} = k_1 \,.\, Q \,.\, C_1 = k_2 \,.\, Q^2\, C_2 = k_3 \,.\, Q^3 \,.\, C_3.$$

From which

$$C_1 : C_2 : C_3 = \frac{1}{Q} : \frac{1}{Q^2} : \frac{1}{Q^3},$$

$$\text{or } p_1 : p_2 : p_3 = 1 : Q : Q^2$$

where Q is the colloid concentration.

Equations (3a), (3b), and (3c), however, represent particular cases of the general equations.

$$\left.\begin{array}{ll} (4a). & n.Na^{+} + 1 \text{ colloid particle} \xrightarrow{(n-)} \text{Colloid} \,.\, (Na)_n \\ (4b). & n.Ca^{++} + 2 \text{ colloid particles} \xrightarrow{(n-)} (\text{Colloid})_2 \,.\, (Ca)_n \\ (4c). & n.Al^{+++} + 3 \text{ colloid particles} \xrightarrow{(n-)} \text{Colloid})_3 \,.\, (Al)_n \end{array}\right\} (4)$$

On equating velocities and proceeding as above we obtain:

$$(4d). \quad p_1 : p_2 : p_3 = 1 : Q^{1/n} : (Q^{1/n})^2.$$

It is at once noticed that the proportionality between the coagulating powers is in the same form as the previous deduction, namely: $1 : X : X^2$, but whereas X had the value $\frac{1}{C_1}$ it has by this deduction the value $Q^{1/n}$ where Q is the colloid concentration.

Deduction of Whetham's Law.

Whetham's law itself is deduced on principles which are comparable at every step with those used in the foregoing deduction (A), and further it is based on practically the same assumptions.

These assumptions are:

(*a*) That the charged colloid is coagulated when its charge is neutralized by a certain minimum ionic charge.

(*b*) That the rate of neutralization, or the probability of the electrical conjugations, is proportional to the concentration of the ions.

(*c*) That the conjugations are electrical only, and are not chemically specific.

Whetham proceeds essentially as follows:

The necessary coagulations occur when $6n$ univalent ions, or $3n$ bivalent ions, or $2n$ trivalent ions, meet 1 colloid particle. (Compare with equations 1a, 1b, and 1c). "The probability of an ion being within reach of a fixed point at any instant is, putting certainty equal to unity, approximately represented by a fraction proportional to the ratio between the volume occupied by the sphere of influence of the ions and the whole volume of the solution, and may be written as $A \,.\, C^1$, where A is a constant and C^1 represents the concentration of the solution," i.e.:

$$(5). \quad \text{Probability} \propto A \,.\, C^1.$$

(We shall write C^1 for any arbitrary concentration and C for the coagulation concentration.)

The probability of the conjugation of n ions at the one fixed point is then given by $(AC^1)^n$. For the same frequency of conjugation of $6n$ univalent ions, or $3n$ bivalent ions, or $2n$ trivalent ions, we should have:

$$(AC_1)^{6n} = (AC_2)^{3n} = (AC_3)^{2n} = \text{constant} = B,$$

where C_1, C_2, and C_3 represent the respective coagulation concentrations.

It is evident then that

$$C_1 = \frac{B^{1/6n}}{A}$$

$$C_2 = \frac{B^{1/3n}}{A}$$

$$C_3 = \frac{B^{1/2n}}{A}$$

From these relations it follows that:

$$(6a). \quad (AC_1)^2 = AC_2, \text{ or } \frac{C_2}{C_1^2} = A, \text{ and}$$

$$(6b). \quad (AC_1)^3 = AC_3, \text{ or } \frac{C_3}{C_1^3} = A^2, \text{ and}$$

$$(6c). \quad p_1 : p_2 : p_3 = 1 : \frac{1}{AC_1} : \frac{1}{AC_2}$$

$$\text{or } 1 : \frac{1}{AC_1} : \left(\frac{1}{AC_1}\right)^2$$

The last relation is in the form $1 : X : X^2$, where $X = \frac{1}{AC_1}$. In the original deduction Whetham does not bring out this inter-relation $\left(X = \frac{1}{AC_1}\right)$ but takes X to be an arbitrary constant. X, however, is known if A can be found, and the latter can be obtained from (5) as follows:

$$AC = \frac{\text{Volume of the spheres of influence of the ions}}{\text{Total volume}}$$

$$= \frac{V}{\text{Total volume}}$$

$$\text{Therefore } A = \frac{V}{\text{Total volume}} \times \frac{1}{C}$$

$$= \frac{V}{\text{Total volume}} \times \frac{\text{Total volume}}{\text{No. of gramme ions}}$$

$$= \frac{V}{\text{No. of gramme ions}}$$

Hence A is the volume of the sphere of influence of 1 gramme ion (in litres).

This will be referred to later in dealing with the experimental figures.

There is, however, one interesting point that has so far been overlooked. The concentrations C_1, C_2, and C_3 are the coagulation concentrations, and we may take them theoretically and experimentally as representing the concentrations at which coagulation is instantaneous; in which case the probability of the conjugations occurring becomes certainty, and hence AC_1 (where C_1 is the coagulation concentration) becomes equal to 1. The proportionality $p_1 : p_2 : p_3$ being equal to $1 : \frac{1}{AC_1} : \frac{1}{(AC_1)^2}$, then assumes the anomalous form $1 : 1 : 1$.

It may be objected that there is no method of detecting instantaneous coagulation, and that there must be a time limit, however small. This is true, and, in fact, there should be a time factor in all probability deductions, owing to the probability of an event occurring being some function of the time. Further, in this particular probability application a time factor is necessary, as the coagulation concentrations are dependent on (and vary with) the time interval over which observations are taken. If a time factor be introduced, and if we then write, "The probability of an ion being within reach of a fixed point," *within a certain time interval*, say, 1 second, or 1 minute, or 24 hours, is proportional to A . C, we obtain by following the usual deduction exactly the same relation:

$$p_1 : p_2 : p_3 = 1 : \frac{1}{AC_1} : \frac{1}{AC_2}$$

Also, as C_1 (and C_2 and C_3) are the concentrations which cause coagulation within that fixed time limit, we obtain in the same way the same anomalous results:

$$p_1 : p_2 : p_3 = 1 : 1 : 1.$$

It therefore appears to the author that the theory of probability can be applied only with certain limitations and only in the following way.

The probability of an ion being within reach of a fixed point within a certain time interval is proportional to the number of such ions, and also to the number of such fixed points, the latter representing (theoretically) the colloid particles. The probability of such a meeting is therefore proportional to the product of the ionic concentration (C) and the colloid concentration (Q). The probability of the simultaneous conjugation (within the fixed time limit) of 6 ions and any 1 colloid particle is then proportional to C^6Q. For the same frequency of conjugation of $6n$ univalent ions, or $3n$ bivalent ions, or $2n$ trivalent ions, we therefore have

$$(7).\quad Q.\,C_1{}^{6n} = Q.\,C_2{}^{3n} = Q.\,C_3{}^{2n} \text{ and}$$
$$C_1{}^{6n} = C_2{}^{3n} = C_3{}^{2n}$$

which is exactly the relation deduced from mass law principles (see equation 1g). That this should be so is evident when we consider, (*a*) that the mass law deductions are based directly on probability principles, (*b*) that in the above case we are concerned with the same fundamental reactions, namely, the conjugation of $6n$ univalent, $3n$ bivalent, and $2n$ trivalent ions with 1 colloid particle, and (*c*) that the frequency of such conjugations directly controls the velocity of the reaction. We have every evidence that the mass law is fundamentally sound, and it is questionable whether probability deductions will give us any more information than the simple application of the mass law. In this particular case the author considers that they do not, but that both lead to the same results as represented by equations (7) and (1g). On treating equation (7) in the same way as (1g) we obtain:

$$\begin{aligned}(8).\quad p_1 : p_2 : p_3 &= 1 : \frac{1}{C_1} : \frac{1}{C_2} \\ &= 1 : \frac{1}{C_1} : \left(\frac{1}{C_1}\right)^2 \\ &= 1 : X : X^2\end{aligned}$$

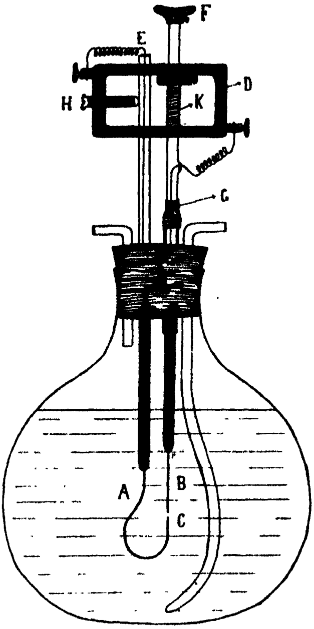
EXPERIMENTAL PART.

A study of the literature shows varying coagulation figures for the same colloid, owing chiefly to differences in the methods of preparation of the colloid, and to its degree of purity or freedom from traces of stabilisers.

Varying figures are also obtained, depending on the time interval before observation and on the method of adding the electrolyte (4, 7, 9, 10). Although the great majority of these figures could be used (and have been used) to show the general weakness of Whetham's law, it was considered advisable, in order to test the points raised in the foregoing discussion, to procure a set of experimental figures for a colloid which was quite free from traces of stabilisers and other impurities. To this end very pure samples of colloidal platinum were prepared, particularly free from foreign electrolytes.

Preparation of Colloidal Platinum.

The platinum sols were made in conductivity water by the Bredig method. For the preparation of the conductivity water a still after the design of Bourdillon (1) was employed, except that the condenser, with its three cooling jackets, was made of boro-silicate glass and all in one piece, having an over-all length of 36 inches. All the glass vessels used, including the tubes in which the coagulations were carried out, were of boro-silicate glass. The conductivity water was siphoned from the collecting vessel into the sparking vessel, always in the absence of CO_2, and immediately before sparking a sample was siphoned over and its conductivity measured. The specific conductivity of the water used in the four separate preparations lay between ·3 and ·5 gemmho. The sparking was carried out with pure platinum poles in the apparatus shown in the figure. The platinum electrodes A and B were sealed into two Pyrex tubes, which passed through a rubber cork. The tube AE fitted firmly into the cork, whilst the tube BF passed through a short but wider tube in the cork. A small piece of rubber tubing at C



supported the tube BF, and at the same time allowed the slight freedom of movement necessary to close the spark gap C. The cork fitted firmly into the mouth of a short-necked 500 cc. Pyrex flask. Both tubes also passed loosely through the hollow ebonite block D. The tube AE was held securely by the screw H, whilst the tube BF had the small spiral spring K attached to it. On compressing F the spark gap C could be closed, and on slightly releasing F the spring K would open C to any desired extent. Inlet and outlet tubes were provided as shown, so that CO_2 free air could be steadily bubbled through the solution during the process of preparation. These tubes also served for the introduction of the conductivity water and for the removal of the prepared sol. The solutions were always prepared in exactly the same way, and as follows: The apparatus was carefully cleaned and the cork put into position. About 450 cc. of conductivity water was then siphoned in, and a sample siphoned into the conductivity cell, and its conductivity measured. The sparking vessel was then placed in a freezing mixture (about —4°), and by manipulating F the sparking was carried on intermittently for about one half-hour with a current of about 6 amps. During the whole process the vessel was gently shaken and the solution kept as near 0° as possible. To this end no arc was held for more than about 10 seconds. After the preparation the sol was siphoned into another flask, and then boiled. It was then set aside for 24 hours, when the pure sol was carefully siphoned over from the precipitate. Generally two successive lots of about 400 cc. each were prepared as above, and then mixed. For the purpose of the experiment it was necessary to be able to prepare comparable samples of the Pt. sol. This was achieved by following the same procedure in each preparation, but making the sols a little stronger than necessary, and then diluting with conductivity water till the boiled sols showed the same specific conductivity. The specific conductivity of the standard sol used in these experiments was 12·5 gemmhos.

The Coagulation Procedure.

The coagulation concentration varies with the time and with the temperature. In the preliminary experiments the observations were taken three minutes after mixing, and in a glass-fronted water bath at 30°, using a magnifying eye-piece, and utilizing as a background a highly illuminated glazed white tile. The black coagulated particles showed up well. This method, however, did not give a sufficiently sharp coagulation concentration, and it was finally utilized only for quick and approximate indications of the necessary concentration of electrolyte required. The method that gave the best results, and which was used exclusively for the results set out in Tables 1 and 2, consisted of mixing the solutions at the room temperature, shaking by hand for one minute, and then putting in the water bath at 30° for 24 hours. It was found that 24 hours served as a convenient minimum time interval, sols uncoagulate after that time remaining stable for at least several days. If necessary, observations were taken with the white background, but generally the time interval of 24 hours was sufficient to allow the coagulated particles to settle completely, and therefore observations became simple.

The coagulation concentration of electrolyte was obtained in the following way: Preliminary experiments having given a fairly close approximation of the required concentration of any particular electrolyte, a range of 6–10 solutions was prepared having electrolyte concentrations varying round this approximate figure. These solutions generally differed from one another by a maximum of 10%, so that the coagulation concentrations obtained in this work can only be considered accurate to within 10%. This is quite sufficient for the purpose in hand, and, in fact, quite apart from the tedious work involved when a considerable number of electrolytes have to be investigated, it is sometimes very difficult to obtain a coagulation concentration within narrower limits. Each of the above solutions was prepared by putting the necessary quantity of electrolyte into a steamed and air-dried Pyrex test tube, adding sufficient water to make ·3 cc., and then rapidly running in 2·5 cc. of Pt. sol. It was necessary to use micropipettes; these were carefully checked, and the percentage error associated with the technique employed was determined. The concentration of the added electrolyte was then so arranged that the percentage error due to the use of these small quantities was always less than 2%. In view of the foregoing, this degree of accuracy was quite satisfactory.

TABLE 1.

Coagulation Concentrations are given in Gramme Ions of Cation per litre, assuming the activity coefficient of the cation to be unity.

Univalent Cations.	Mean coag. conc.	Mean coag. power.	Relative coag. power.
Na Acetate	·010	100	1
$\frac{1}{2}K_2Cr\,O_4$	·0095	105	1·05
KNO_3	·0081	123	1·2
KCN	·0058	172	1.72
NaCl	·0048	208	2·1
KCl	·0036	278	2·8
$RbNo_3$	·0022	455	4·5
$CaNo_3$	·0022	455	4·5
NH_4NO_3	·0021	476	4·8
NH_4Cl	·0015	667	6.7
$\frac{1}{2}K_2Cr_2O_7$	·0014	714	7·1
$AgNO_3$	·00032	3,120	31

	Mean coag. conc.	Mean coag. power.	Relative coag. power.
Bivalent Cations.			
$CaCl_2$	·00013	7,690	77
$Ca(NO_3)_2$	·00012	8,330	83
$BaCl_2$	·000072	13,900	139
Z_nCl_2	·000062	16,100	161
$CuSO_4$	·000026	38,500	385
$CuCl_2$	·000018	56,000	560
Trivalent Cations.			
$FeCl_3$	·0078	128	1·28
$\frac{1}{2}Fe_2(SO_4)_3$	·000040	25,000	250
$LaCl_3$	·000011	90,900	909
$\frac{1}{2}Al_2(SO_4)_3$	·0000087	115,000	1,150
$AlCl_3$	·0000080	125,000	1,250
$Y(NO_3)_3$	·0000079	127,000	1,270
$\frac{1}{2}Di_2(SO_4)_3$	·0000067	150,000	1,500
$Ce(NO_3)_3$	·0000066	152,000	1,520

Note on Table I.

The mean coagulation concentrations only are given, and represent the mid-values between pairs of concentrations, one of which is just insufficient to produce coagulation and the other just able to bring it about. As has been pointed out, these mean values are liable in general to a maximum error of 10 per cent.

TABLE 2.

Effect of Altering the Colloid Concentration.

	1		2		3	
	a Coag. conc. of electrolyte.	b Relative coag. power.	a Coag. conc. of electrolyte.	b Relative coag. power.	a Coag. conc. of electrolyte.	b Relative coag. power.
Univalent Cations.						
NaCl	·0048	2·1	·0061	1·6	·0079	1·3
KCl	·0036	2·8	·0047	2·1	·0062	1·6
$RbNO_3$	·0022	4·5	·0031	3·2	·0037	2·7
$CsNO_3$	·0022	4·5	·0026	3·8	·0032	3·1
NH_4Cl	·0015	6·7	·0015	6·7	·0015	6·7
$AgNO_3$	·00032	31·3	·00032	31·3	·00034	29·4

	1		2		3	
	a	b	a	b	a	b
	Coag. conc. of electro-lyte.	Relative coag. power.	Coag. conc. of electro-lyte.	Relative coag. power.	Coag. conc. of electro-lyte.	Relative coag. power.
Bivalent Cations.						
$Ca(NO_3)_2$	·00012	83	·00012	83	·00012	83
$BaCl^2$	·000072	140	·000075	133	·000075	133
$CuSO_4$	·000026	385	·000026	385	·000024	417
Trivalent Cations.						
$Al_2(SO_4)_3$	·0000089	1,120	·0000078	1,280	·0000062	1,610
$AlCl_3$	·0000080	1,250	·0000069	1,450	·0000060	1,670
$Y(NO_3)_3$	·0000081	1,230	·0000067	1,480	·0000052	1,920
$Ce(NO_3)_3$	·0000063	1,590	·0000050	2,000	·0000050	2,000

Column 1 gives the coagulation concentrations and relative coagulating powers of the various electrolytes using the standard Pt. sol.

Column 2 gives the corresponding figures when the Pt. sol has been diluted to ·6 of its original strength.

Column 3 gives the corresponding figures when the Pt. sol has been diluted to ·4 of its original strength.

An examination of Table 1 shows large variations amongst the coagulating concentrations of cations of the same valence. In general, similar variations are shown with most colloids (3, 5, 6, 7, 12), and it is sometimes concluded that these variations alone are sufficient to condemn Whetham's law. However, the wide differences in coagulating powers of uni-, bi-, and trivalent ions far outshadow these lesser variations, and the Hardy-Schultz qualitative law stands out. It is instructive, therefore, to neglect the more exceptional cases in the table (e.g., $AgNO_3$ and $FeCl_3$), and to examine the foregoing deductions in terms of a standard representative of each of the valence series. To this end we shall take KCl as representative of the univalent series, $BaCl_2$ of the bivalent, and $AlCl_3$ of the trivalent. As these have the same anion (namely, chlorion), the comparison is apt. It is, of course, recognized that the general conclusions must be very approximate, but as will be seen, the theoretical and practical results are not only far from approximate agreement, but they vary so enormously as to demand the conclusion that the theoretical basis is unsatisfactory.

The foregoing mass law deduction (A), and also Whetham's law when deduced on comparable lines, lead to the proportionality:

$$p_1 : p_2 : p_3 = 1 : \frac{1}{C_1} : \frac{1}{C_2}$$

$$\text{or } 1 : \frac{1}{C_1} : \left(\frac{1}{C_1}\right)^2 \quad \text{(equations 1k and 8).}$$

The coagulating concentrations C_1, C_2, and C_3 of KCl, $BaCl_2$, and $AlCl_3$ are respectively ·0036, ·000072, and ·000008, and the respective coagulating powers 2·8, 139, and 1,250 (Table 1). The proportionality experimentally obtained is therefore

$$(9a).\quad p_1 : p_2 : p_3 = 1 : 50 : 450.$$

The proportionality using the above theoretical relations is, however,

$$(9b).\quad 1 : 278 : 14{,}000 \left(\text{Using } 1 : \frac{1}{C_1} : \frac{1}{C_2}\right)$$

or

$$(9c).\quad 1 : 278 : 77{,}000 \left[\text{Using } 1 : \frac{1}{C_1} : \left(\frac{1}{C_1}\right)^2\right]$$

The experimental relation (9a) differs so seriously from the theoretical (9b) and (9c) that we must conclude that the theoretical proportionality does not give any quantitative indication of the relative coagulating powers.

Whetham's constant A has been shown to be the volume of the sphere of influence of a gramme ion, and in the ordinary deduction is taken as constant for all ions. If C_1, C_2, and C_3 represent the coagulation concentrations, then, as the probability of the necessary conjugations occurring is now certainty, the product of the volume of the sphere of influence of each ion multiplied by its concentration must in each case become equal to unity. Hence AC_1, AC_2, and AC_3, for the salts KCl, $BaCl_2$, and $AlCl_3$, are all equal to 1, and therefore A, which is assumed to be constant, varies from 278 to 14,000 to 125,000.

In the foregoing deductions it is assumed that in all cases the given colloid is being used at constant concentration, and therefore in the deduced proportionalities the term expressing this concentration does not appear. It is evident, however, that the probability of the occurrence of the necessary conjugations or the velocity of coagulation must be directly proportional to Q, the colloid concentration. This means that the coagulating power of any cation should be proportional to Q. Table 2, which gives the coagulating concentrations and the coagulating powers at varying colloid strengths, shows that this is by no means the case. With univalent cations the coagulating power does decrease in general with decrease in colloid concentration, but a direct proportionality does not exist. With bivalent cations the coagulating power seems to be definitely independent of colloid concentration, and with trivalent cations the coagulating power actually increases with decrease in colloid concentration. Similar results are obtained by Kruyt and van der Spek (8), using other colloids. It is evident that such variations must cause different coagulation proportionalities to be obtained according to the strength of colloid used. Using KCL, $BaCl_2$, and $AlCl_3$ from Table 2, the coagulating powers of the standard platinum sol are 1 : 50 : 450, whilst the corresponding powers when the sol is diluted to ·4 of its original strength are 1 : 83 : 1,040. Burton and Bishop (2) using arsenious sulphide sol over a much wider range of dilution, show a correspondingly wider range of variation in coagulating powers. Such results are obviously in direct contradiction to the theoretical requirements.

Deduction (B) in the foregoing theoretical treatment leads to relation (4d), namely, $p_1 : p_2 : p_3 = 1 : Q^{1/n} : (Q^{1/n})^2$. The actual value of n is unknown, but from equation (4a) it is at once evident that n must always be integral and greater than 1. Further, the colloid concentration, Q, is generally only approximately known; but the colloid solution can always be diluted until Q is less than unity. When such is the case, $Q^{1/n}$ is obviously less than unity; and the relation $p_1 : p_2 : p_3$ becomes a decreasing proportionality. This makes the univalent ions the strongest coagulating agents and the trivalent the weakest. As this is in direct contradition to all experimental results, deduction (B) must be inherently incorrect.

In conclusion, it should be mentioned that solutions of colloidal platinum have been found by the author to be definitely acid. The standard sols used in this work (specific cond. 12·5 gemmhos) showed by direct conductivity, and also by conductometric titration, an acid normality of ·00004. The evidence definitely indicates a surface ionization, and in a later paper the author will attempt to show that the coagulation of colloidal Pt. is due to a surface salt formation.

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INFECTIOUS NECROTIC HEPATITIS OF SHEEP IN VICTORIA. A BRAXY-LIKE SHEEP DISEASE

by

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During an investigation into braxy-like diseases of sheep in Victoria, the author encountered a condition which bore marked resemblances to Black Disease (Dodd), and for which the name Infectious Necrotic Hepatitis is proposed.

Previous Investigations. As far as Australia is concerned, detailed investigation into the braxy-like diseases of sheep has been confined to a few workers.

In 1910 Dr. J. A. Gilruth (4) published the results of his researches into the etiology of a disease occurring in Victoria and Tasmania, thought by him to be the same in both States, although the organism which he considered the etiological factor differed slightly in the two cases. In Victoria and Tasmania the disease occurred only in the dry weather of summer, practically ceasing after the onset of the general autumnal rains. It was confined to young sheep (2–2½ years) in good condition, males and females being affected alike. The symptoms were rarely observed, and were confined to a general dullness and disinclination to move. As a general rule, however, animals were found dead early in the morning, after being apparently normal the preceding night. The post-mortem appearances were considered characteristic. Patches of gelatinous subcutaneous oedema, sometimes blood-tinged; serous cavities containing a variable quantity of clear or blood-stained watery exudate; stomach (abomasum) more or less deeply congested, but as a rule not ulcerated; liver showing mottled areas of necrosis; spleen slightly enlarged and softer than normal, kidneys congested, and lungs oedematous; sometimes subendocardial haemorrhages were present; lymph-glands more or less oedematous and haemorrhagic.

He states that "in animals dead for some hours the characteristic bacilli are present in the effusions, organs, or even in the blood, but never in a state of purity. They are most numerous in the peritoneal effusion, but invariably associated with other organisms—some long, thin, and filamentous, others broad and large, both sporulating, and probably of intestinal origin and putrefactive, while cocci may also be present." He points out that the peritoneal effusion is very likely to be contaminated with putrefactive organisms if examined some time after death, on account of the sheep's carcase forming an excellent incubator for organisms, owing to insulation by wool and subcutaneous fat, thus enabling the bacterial flora of the large intestine to traverse the intestinal wall and reach the peritoneal fluid.

Gilruth attempted to purify his cultures by repeated passage through small laboratory animals or through sheep. This suggests that he considered one pathogen to be present in the original material. Such fluids as the peritoneal effusion of a sheep dead only a short time would be most likely to contain such pathogenic organisms as *Vibrion septique*, *C. sporogenes*, etc., and although there is no reason to doubt that the original pathogen could gain the peritoneal fluid, together with other bacteria, yet it is extremely likely that even after passage through guinea-pigs, the resultant material would contain a mixture of organisms. It is even possible for the original pathogen to be lost by passage. For instance, the organism isolated by the writer does not as a rule enter the blood-stream. If a culture of this organism contaminated with *Vibrion septique* be inoculated into a guinea-pig, and the heart blood cultured at the time of death, the result would almost certainly be a pure culture of *Vibrion septique*. It is highly probable that Gilruth was working with impure cultures, and assuming that the causal organism was present in the original material, it is possible that it may have been lost during the subsequent experiments.

It is interesting to note here that apart from the bacterial content of the peritoneal fluid and the wall of the abomasum, Gilruth's cases correspond very closely to those examined by the writer during the last two years (see later).

In March, 1921, Dr. S. Dodd (2) published an article on "Black Disease" in New South Wales. He concluded, after the examination of one hundred cases of this disease (all examinations being made on sheep killed while sick, or post-mortemed immediately death had taken place), that the primary lesion was an hepatic necrosis, and that all other lesions were secondary, and due to the action of the toxin produced by the bacilli in the liver and circulated in the blood-stream. He definitely determined that at the time of death organisms are confined to the hepatic lesions, and cannot be found elsewhere in the body, although in rare cases bacilli were cultured from the liver when no necrotic foci could be found. These he considered mass infections. He found the mucosa of the abomasum frequently congested, often in patches round the pylorus; infrequently circular ulcers of a superficial character were present in the mucosa of the abomasum, but these were sterile, and according to Dodd, of a peptic nature and a secondary character.

Another important observation made by Dodd was that all cases were infected with the liver-fluke *(Fasciola hepatica)*, and he advanced the theory that the liver-fluke is probably the mechanical carrier of the causal organism to the liver. Facts supporting this theory are the seasonal incidence of the disease, the geographical features of the infected paddocks, and the universal occurrence of fluke infection in cases of "black disease." In New South Wales the disease is seasonal, occurring from late summer to early winter. Dodd suggested that black disease is probably identical with the braxy-like diseases of Victoria and Tasmania.

In Western Australia a disease of sheep occurs which simulates braxy, and which is known locally as "Beverley disease." It is confined to a fertile region in one of the oldest cultivated areas of the State. It has not been recorded from the wetter and colder parts, and is unknown in the long, dry summer, when feed is scarce, but commences in the autumn, with the first growth of herbage after the autumnal rains. Small outbreaks are the rule in winter months, adjacent farms being frequently simultaneously affected, but the first big outbreaks occur in the spring, "at the time of the spring transitional period." The disease is seasonal, resembling braxy in Europe. According to Dakin (1) sudden and unsuspected death is typical. He describes two types of symptoms, one characterized by unconsciousness and quiet death, the other by convulsions, head retraction, with death in 2–3 up to 24 hours, or even after several days. In many cases there is evidence of the sheep having scoured. The carcase is invariably that of a well-nourished sheep.

Post-mortem appearances described are mainly negative. The kidneys "present a marked lesion, being quite soft, sometimes almost fluid." There is little or no trace of inflammation of the intestines, and "certainly no necrosis of the mucous membrane." Lambs seem to be mainly affected, although losses amongst two-tooth sheep are common, and older sheep have suffered on some properties. Dakin concludes that the local disease in Western Australia is braxy, but that the organism supposed to be the cause in Scotland, Ireland, and Iceland was not the cause in Western Australia. Furthermore, in sheep examined immediately after death, *no organism could be discovered which could be regarded as the cause of the disease.* However, he states that "there was evidence that the disease was of bacterial origin, but the condition of the feed, and the treatment of the sheep in regard to it, seemed to be associated factors of great importance." No mention is made of the liver, examined either macroscopically or microscopically; the only bacteriological examination mentioned was of peritoneal exudate, which was found to be sterile.

Disease in Victoria. Two outbreaks of the disease in sheep were investigated by the present writer, one in 1921 and the other in 1922. These outbreaks occurred on two farms in the Goulburn Valley district in Victoria, where, judging by the history of the disease as elicited from the local farmers, it had been endemic for several years prior to 1921.

Occurrence. In both years (1921 and 1922) the deaths commenced in the late summer, and continued until the first heavy rains of winter (which in 1921-1922 did not occur until July and August in this district). No deaths occurred in the spring and early summer.

Symptoms. As a rule death is quite sudden, and the period during which symptoms are shown is so short as to be missed unless specially looked for. Sick sheep may be detected, as they lag behind the rest of the flock, being the last to rise when moved and the first to lie down. Later they assume a recumbent attitude, breathing becomes stertorous, and death usually follows within half an hour.

Post-mortem Appearances. Unless sheep are examined immediately after death putrefactive changes tend to mislead, and a false description of lesions is likely to result. Subcutaneous vessels may be injected—excess of peritoneal fluid, either clear or slightly blood-stained. Intestinal vessels injected—mucosa of the abomasum slightly reddened, but no sign of ulceration, gall bladder distended with bile. Pericardial sac distended with a clear fluid, coagulating on exposure to air, lungs and kidneys congested.

Liver shows the presence of irregular areas of necrosis, the necrotic tissue yellowish, grey, and friable. In at least two cases no trace of fluke infection could be detected.

Bacteriology. Cultures made from necrotic liver tissue were positive, that is, bacterial growths were obtained from them. All other cultures were negative, both aerobically and anaerobically. Anaerobic cultures of necrotic liver furnished a growth of a bacillus, apparently in pure culture.

Cultural Characters of the Bacillus. The original cultures were sown into bullock's heart medium (cooked meat medium). In this the organisms grow rather slowly, no gas formation being noticed as a rule earlier than 24 hours' incubation; 24–48 hours shows the commencement of gas formation, with slight reddening of the meat at the base of the tube. The medium is rendered acid. Cooked liver medium (using minced sheep's liver instead of bullock's heart), to which 2% peptone is added, produces the best growth for this organism. With this medium gas formation is usually marked at 18 hours, and a more vigorous and plentiful growth takes place.

The morphology of the organism varies according to the reaction of the medium and the rate of growth. In a rapidly growing culture, sown into cooked liver medium, titrated to about pH 7·2, the organism appears as a short, stout rod, with distinctly rounded ends, occurring singly, in pairs, or in short chains of three or four elements. In a slowly growing culture the individual elements are longer. As a rule sporulation does not begin until after 18 hours' incubation, although in some cases numerous sporulating forms will be seen if an 18-hour-old culture is examined. In media whose reaction is about pH 6·0 the bacilli appear longer, more slender, with a tendency to filamentous growth (up to 30μ in length), and show no tendency to sporulate.

Spores. These are large, distinctly oval, only slightly distend the bacillus, and are situated either terminally or subterminally. An occasional central spore has been observed. The addition of 2% peptone to the media seems to favour sporulation.

Staining. The organism is Gram-positive in early cultures, and *in situ* in the liver of natural cases of the disease. It rapidly loses the power of retaining the Gentian violet. A marked peculiarity of the bacillus is the beaded or vacuolated appearance of the organisms when stained by any method. This has no relation to sporulation.

Motility. The bacillus is non-motile on the slide, in a hanging-drop preparation.

All cultures degenerate quickly; this is especially seen in a rapidly growing culture. Examined after 48 hours, a culture will show mainly spores and ghost forms.

Broth. Unless every precaution is taken to ensure the medium being strictly anaerobic, cultures cannot be grown in broth: 2% peptone liver broth under vaseline has been used with moderate success. Tubes should be cooled rapidly after they are removed from the autoclave, and inoculated immediately. If left more than two days before being inoculated sufficient oxygen is absorbed from the air to prevent growth, and it seems impossible to drive this off by simple boiling. In addition care must be taken to use broth of a pH 7·6. (In inoculating broth under vaseline the following method was found to be satisfactory: Pasteur pipettes were constricted about ½ inch from the slender tip: sufficient inoculum was then taken from the seed culture, the pipette was pushed through the vaseline cap, and the tip of the pipette broken off by gentle pressure against the side or bottom of the tube. The hole in the vaseline cap left after the withdrawal of the pipette was then closed by heating the culture tube at the level of the vaseline until the latter began to melt. The melted paraffin then flowed and closed up the hole. The tube was then placed in cold water to hasten the solidification of the vaseline. A similar method was adopted to withdraw culture for microscopical examination.)

The growth in broth appears as a uniform cloudiness in 18 hours. Clearing and auto-agglutination commences after this, and is usually complete by the end of the second day. As a rule no gas is formed, though on one or two occasions a few gas bubbles have appeared after a little broth has been withdrawn for examination. No spores have ever been seen in broth cultures.

Agar: Surface Culture. On no occasion have I been able to grow this bacillus as a surface culture. Different media, such as blood agar, serum agar, and glucose agar, have been tried in McIntosh and Fildes' jar, and with pyrogallic acid, but with no sign of growth. Whether this is due to imperfect anaerobiosis or to deficiency in the media it is impossible to say.

Shake Culture. In 3% glucose agar growth has occasionally been accomplished. The colonies form in the depths of the media as woolly growths without a nucleus. No gas formation or shattering of the media is evident.

Brain Medium. The growth in this medium is slow: no blackening occurs, and only sluggish sporulation.

Alkaline Egg Medium, Serum Water, Sugar Media (except 3% glucose agar). No growth.

The organism appears to be an anaerobe of the strictest type, with fastidious reaction requirements.

Pathogenicity. The organism is extremely pathogenic for guinea-pigs, rabbits, and sheep. The effect on other animals has not been tested.

Guinea-pigs. Subcutaneous injection of 0·05 cc. of an 18-hour broth culture kills a guinea-pig in 24–36 hours. Slightly larger doses of meat cultures are necessary to kill in the same time. Several hours after inoculation the guinea-pig shows general malaise, and on examination a large swelling on the inoculated side can be felt. This usually extends from the inoculated groin forward, in some cases to the axilla of the same side, or even extending over the whole abdominal surface. The post-mortem appearances are typical. Extending from the point of inoculation forwards, the subcutaneous tissues are engorged with a watery oedema, slightly blood-tinged in the region of the inoculation point, but clear elsewhere. This exudate coagulates on exposure to air. Sometimes the subcutaneous tissues are nearly half an inch in thickness. There is no gas formation or emphysema or odour. The muscle of the leg may be paler than normal.

The intestines, especially the small intestines, show congested blood-vessels and general reddening, but there is no increase in the amount of peritoneal fluid; the spleen is normal. The liver may show one or two areas of necrosis under the capsule which are sterile and of toxic origin, and with few exceptions the gall bladder is found distended with bile. The organs of the thorax are normal, although there may be an increase in the amount of pericardial fluid. Cultures can usually be recovered from the subcutaneous oedema at the point of inoculation, but frequently those from a distance prove sterile. Smears of the subcutaneous exudate show very few organisms. Sometimes several fields are examined before one is seen. Other body fluids are sterile.

Sheep. Inoculation of 0·25 cc. or more of culture kills sheep in 36–48 hours. (Smaller doses have not been tried.) At 24 hours they show a definite lameness in the injected leg, which is swollen and painful. Later rumination ceases, the animal assumes a recumbent attitude, and dies quietly. Post-mortem appearances are on the whole similar to those of the guinea-pig. The oedema of the subcutaneous tissues is very extensive. No necrosis of the underlying muscles has been observed. In the abdomen there may be a slight excess of peritoneal fluid, the intestines are injected, and the mucosa of the abomasum shows congestion, which may be very marked. No necrosis of the liver has been observed, and the gall bladder is distended with bile. Both the liver and the kidneys are congested.

In the thorax, pericardium is distended with a pale clear fluid, which coagulates on exposure to air. The lungs and heart are normal. The heart-blood and fluids from the serous cavities prove sterile on culture. Organisms are not plentiful in the subcutaneous oedema, but can be recovered by culture.

Rabbits. Post-mortem appearances differ in these animals in that the oedema is not so marked.

Immunization Experiments. At the outset it was hoped to discover some method of immunizing sheep against this disease, preferably a method having a maximum of immunizing power and a minimum of risk. We decided to try the efficacy of germ-free preparations of the organisms, such as culture filtrates and aggressins (cf. Blackleg), and also killed vaccines. It was decided to eliminate these methods as useless before trying the effect of preparations such as attenuated live cultures. Although in such cases the process of attenuation may be generally successful, yet the introduction of live spores into animals is always accompanied by a certain amount of risk, due to the fact that some individuals may have a lowered resistance, or that in spite of the process of attenuation some spores retain a sufficient degree of virulence to produce illness, or even death. This is exemplified with some anthrax vaccinations, where, although carelessness in the introduction of vaccine can be excluded, yet a sufficiently large percentage of mortality takes place among the vaccinated animals to discredit the use of the preparation. In this connection mention might be made of the method of immunization by the introduction of live culture by the digestive tract. Gilruth produced an active immunity against the organism which he isolated from sheep by this method. Although no serious effects are produced in the animals themselves as a result of this method, yet it is highly probable that when a sporing culture is used, organisms *would pass through the digestive tract*, and thus bring about a further contamination of pasture, the very thing to be avoided where natural infection probably takes place by the ingestion of pathogenic organisms along with the food.

Bearing in mind the success with which sheep and calves can be immunized against blackleg by a germ-free culture filtrate of *B. chauvoei*, we decided to try the efficacy of a similar product in immunizing against the experimental disease produced by inoculation of the organism into guinea-pigs.

Two series of experiments were conducted in order to test the efficacy of culture filtrate as an immunizing agent. The medium used for the production of the filtrates was cooked liver medium, the second series differing from the first in that in the former 2% peptone was added to the medium.

Cultures of the organism were incubated for varying lengths of time, ranging from 2–14 days, then filtered, firstly through cotton-wool and filter-paper, and finally through a Chamberland F. candle. In some cases the culture was diluted before filtration. In each case the filtrate was tested for sterility before use. Six culture filtrates were prepared, but from the experiments conducted with them it was evident that no immunity could be established by the inoculation of doses of 1–2 cc. into guinea-pigs.

Vaccines Killed by Heat. Eighteen-hour broth cultures of the organism were centrifuged, and the organisms so deposited were emulsified in sterile saline and subjected to a heat of 63°C. for 30 minutes, such a heat being sufficient to kill the vegetative forms of the organisms. Such vaccines were used in varying doses, but no immunity could be induced by the use of these preparations.

Aggressin Experiments. Two sheep were inoculated with 1 cc. of a cooked liver medium culture of the organism. *Expl. Sheep 5* was killed while moribund; *Expl. Sheep 7* was allowed to die, death taking place in 36 hours. The subcutaneous exudate in the inoculated thigh was collected as cleanly as possible and placed in sterile jars. This fluid partly coagulated on standing. It was then filtered through cotton-wool, the fluid from the coagulum being expressed by pressure. The filtrate was then passed through a Chamberland F. candle and put into amber-coloured sterile bottles. This germ-free filtrate proved very toxic on inoculation into guinea-pigs.

From Expl. Sheep No. 5 0·075 cc. was found to be the M.L.D. for guinea-pigs of about 500 grm. weight.

From Expl. Sheep No. 7 0·03 cc. was found to be the M.L.D.

Inoculations of 0·005 cc. into guinea-pigs produced marked symptoms and large swellings at the site of inoculation, but recovery resulted when such doses were used. No immunity resulted from inoculations with quantities under the M.L.D. (This was also the case when guinea-pigs recovered from an injection of live whole cultures.)

Toxin Production.

The toxicity of this fluid obtained from the sheep led to experiments to determine if under any conditions cultures could be obtained which on filtration would prove as toxic as this fluid. Previous experiments with culture filtrates showed that in one culture incubated for 48 hours the filtrate was toxic enough to produce death in doses of 1·5 cc.; incubation for periods longer than 48 hours yielded a non-toxic filtrate. (This is to say in doses of 1–1·5 cc.)

Filtrates were made of two cultures, one in broth, the other in peptone liver medium, after incubation for 18 hours. Both these filtrates proved toxic in doses approximating to the M.L.D. of the exudate from sheep, the filtrate from the broth culture proving the more toxic of the two. With the filtered exudate from sheep, and the filtrate from the 18-hour cultures, the post-mortem appearances of inoculated guinea-pigs are identical, and show a marked similarity to the condition produced in these animals by the inoculation of whole culture, the difference being the sterile nature of all fluids of the body. The gelatinous subcutaneous oedema is well marked, there is slight injection of the subperitoneal vessels of the intestine, the gall bladder is usually distended, and sometimes necrotic areas are present in the liver. Experiments were then undertaken to produce an "anti-toxin" to the toxin formed in the cultures and to the toxic filtrate of the exudate from sheep. The first series of experiments proved fruitless. Guinea-pigs were inoculated with very small doses of toxin, and the doses were increased slightly every 7 days. It was found, however, that when the minimum lethal dose was reached the guinea-pigs succumbed, having derived no immunity from the inoculation of smaller doses. Further experiments on antitoxin production proved negative.

General Discussion of Experimental and Field Work. Although only a few cases of the natural disease have been investigated, there is no doubt that sheep in Victoria are affected with a condition resembling closely, if not identical with, black disease described by Dodd. Whether the disease has been introduced from New South Wales it is impossible to state, but there was no evidence of the sheep on either property having been brought from New South Wales, nor had sheep ever come from this source, as far as the owners could remember.

Judging from the post-mortem findings alone, the diseases would appear to be identical, the lesion in each case being an hepatic necrosis caused by an anaerobic bacillus.

Dodd considers that the liver-fluke is the mechanical carrier of the causal organism from the bowel to the liver. Recent work on the life history of the liver-fluke shows that the common route of the young fluke from the intestine to the liver is *via* the intestinal wall and peritoneal cavity, entering the liver through the peritoneal coat, and thence to the smaller bile-ducts. It is possible also that some may gain the liver by the portal blood-stream. What Dodd means by the "mechanical" carrier is hard to say, except on the supposition that young flukes enter the liver *via* the bile ducts. With our present knowledge of the method of infection by liver-flukes, one would expect to find peritoneal infections as well as liver infections if the fluke is the carrier of the organism. This is never found in natural cases. If it be a fact that black disease and fluke infection go hand in hand, another explanation of the relation of the liver fluke to the disease is suggested. It is possible that the presence of the parasites in the liver, with the consequent destruction of hepatic tissue, may produce a suitable nidus for the growth of the organisms which have gained the portal circulation, and any condition which has a deleterious effect on the liver cells, at the same time lowering the resistance of the tissues, would probably lead to infection, e.g., other migrating parasites, such as *Cysticercus tenuicollis.*

The disease described by Gilruth is apparently the same as that which is present in New South Wales and Victoria at the present time. Apart from the bacterial content of the body fluids, which can be considered the result of post-mortem invasion, the necrosis of the liver stands out as the main lesion; in fact, by omitting from Gilruth's description of cases those conditions which can be ascribed to post-mortem changes, the similarity is remarkable, amounting almost to proof of identity.

As Gaiger (3) has definitely determined that braxy is a disease of sheep characterized by a bacterial necrosis of the mucosa of the abomasum, the term braxy should rightly be used for such a disease, and no other. In the past all diseases of sheep which have had the one point in common, namely, a sudden and quiet death, have been grouped together as braxy, and hence much confusion now exists with regard to these diseases.

The Australian disease is seasonal, and the seasons of its occurrence are apparently the same both in Victoria and New South Wales, i.e., at the end of the summer, being limited or checked by the onset of the autumnal rains. It occurs when the country is dry and feed is scarce. During such times the close-cropping of sheep in their endeavour to find food is accompanied by the ingestion of a quantity of soil, and the appearance of the disease may possibly be explained in this way. With the onset of good rains, and a consequent luxuriant growth of grass, the possibility of ingestion of soil becomes more remote, and the disease slackens off or disappears altogether until the following summer. The organism isolated by Dodd bears a strong resemblance to the organism isolated by the writer in Victoria, the main difference being the extreme difficulty with which the latter organism is cultivated. Dodd has produced cultures in milk, alkaline egg medium, and brain broth, and also stab cultures in agar, whereas no cultures have been obtained in these media by the writer. Also Dodd found liquid paraffin adequate on the surface of his media in preserving anaerobic conditions, but this material was found to be useless in the case of the Victorian organism.

Histology of the Liver Lesions. The lesions in the liver of affected sheep have been in each case histologically identical. These consist of an irregular necrosis of hepatic tissue. The shape and appearance of the lobules of the liver are maintained in the necrotic area, but the individual cells are smaller, non-nucleated, and irregular. The necrotic tissue is bounded by an irregular zone of tissue reaction. This is made up of a dense mass of leucocytes, both small-celled and poly-nuclear types being present. Immediately inside this zone can be seen the bacilli associated with the lesion. They are present in large numbers, the majority lying immediately adjacent to the zone of reaction, but others are scattered throughout the necrotic tissue. The bacilli appear singly, in pairs, and in fairly long filaments, and here and there a sporing element can be seen. Outside the reaction zone the liver capillaries are engorged with blood, and the liver cells show cloudy swelling. Away from the lesion the liver is congested, but otherwise shows no apparent change in histology.

In addition to these necrotic areas in the liver, in several cases examined other pathological processes have been seen, which apparently bear no relation to the necrotic process. They consist of areas of leucocytic aggregation, composed largely of eosinophiles, the remainder being polymorphonuclear leucocytes. Exactly what the nature of these foci is has not been determined, but the high eosinophile content would suggest that they were parasitic in origin, and probably associated with fluke infestation.

The organism isolated from natural cases of the disease in Victoria has been compared with other anaerobes in this laboratory, but it cannot be considered to resemble any of these very closely. There are, however, some points of resemblance between it and *B. oedematiens* (Weinberg), as described by different American workers. It differs, however, in point of size and in the reluctance with which it grows in many of the stock types of media.

SUMMARY.

1. There is present in Victoria a disease of sheep similar to black disease as described by Dodd, and distinct from braxy of Scotland.
2. The main and primary lesion is an hepatic necrosis, due to infection with an anaerobic bacillus.
3. The method of natural infection is unknown.
4. The bacillus isolated in Victoria resembles *B. oedematiens* (Weinberg) in a number of its characteristics.
5. Recovery from artificial infection with the Victorian organism produces no immunity.
6. Aggressin and culture filtrate have no immunizing power.
7. The organism produces a toxin in 18-hour-old culture filtrates, which decreases on further incubation, and is virtually absent after three days.

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A COMPARISON OF THE UTILIZATION OF NUCLEIC ACIDS OF ANIMAL AND VEGETABLE ORIGIN

by

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OBJECTS OF THE EXPERIMENTS.

For some years past investigations have been proceeding in the Department of Biochemistry and General Physiology with the object of ascertaining the effects of administration of nucleic acids of animal and vegetable origins upon the growth and life-history of animals. One result of these experiments, which will be described in a subsequent communication, has been the discovery that certain modifications in the life-history of the animals which are induced by the administration of nucleic acid differ in quantity, if not in kind, with the source, vegetable or animal, of the nucleic acid. This fact suggested the possibility that nucleic acids of animal and vegetable origin may be utilized by animals in different ways. If both forms of nucleic acid were broken down into their constituent phosphoric acid, carbohydrate, and nitrogenous moieties in the alimentary canal, then, it is true, one would anticipate little, if any, difference in their effects upon the animal, for the pentose yielded by vegetable nucleic acid would be substantially equivalent to the hexose yielded by animal nucleic acid, so far as utilization in metabolism is concerned, and otherwise the products of decomposition of the two nucleic acids would differ only in the substitution, among the products derived from animal nucleic acid, of methyl-uracil (thymine) for the uracil which is present in nucleic acid of vegetable origin (13).

So far as the final products of decomposition are concerned, therefore, the two types of nucleic acid might be expected to be virtually equivalent to one another. According to Abderhalden (1), however, the breakdown of nucleic acid in the alimentary canal ceases with the formation of nucleotides and nucleosides, the latter being absorbed directly into the bloom-stream (5), where they are thereafter demonstrable (20). If this be the case, and if nucleic acid were utilizable, wholly or in part, in the form of nucleotides, or even as nucleosides, then in so far as this type of utilization is concerned the two substances would no longer be equivalent, for nucleotides of vegetable origin cannot be converted into nucleotides of the animal type without preliminary decomposition, to permit the insertion, in the middle of the molecule, of a hexose molecule in place of a pentose. Thus if any appreciable proportion of nucleic acid can be utilized in an undecomposed or partially decomposed form, nucleic acids of animal and vegetable origins should differ to that extent in their utilizability by the animal.

There are not wanting evidences of an inconclusive but suggestive character. that such a difference of utilizability may actually subsist between nucleic acids of animal and vegetable origin. Thus Jones (12) has shown that the pancreas of the pig contains an enzyme capable of decomposing plant nucleic acid into its nucleotides, while it does not split animal nucleic acid. Levene and Medigreceanu (15) have found that whereas both types of nucleic acid are split by enzymes contained in the digestive juices, yet animal nucleic acid is split very much more slowly than nucleic acid of vegetable origin. Ringer and Underhill (19) have shown that vegetable nucleic acid when administered intravenously elicits symptoms of shock, with evident upset of nitrogenous metabolism, while animal nucleic acid, similarly administered, does not elicit any such response. In other words, vegetable nucleic acid, as such, behaves in the circulation like a foreign substance, while animal nucleic acid is tolerated without any appreciable disturbance of metabolism.

Numerous studies have been made of the effects of administering nucleic acid by mouth to human beings. These studies have been summarized by Givens and Hunter (10), but it is significant that in their summary no distinction is drawn by these authors between the two types of nucleic acid, although some of the authors whom they cite employed the one type of nucleic acid and some the other. The majority of investigators have confined themselves to ascertaining the effect of nucleic acid administration upon the uric acid output of the subjects. A few have extended the scope of their analyses to include the total nitrogen and urea output. There does not appear heretofore to have been any attempt to ascertain the effect of the administration of nucleic acid upon the output of other important metabolites or upon the properties of the urine. The doses administered have usually been comparatively small; the largest amount administered in any of the experiments quoted by Hunter and Givens having been a total of 50 grms. of a very impure preparation (as its purine content shows), administered by Frank and Schittenhelm (9) throughout the duration of an experiment lasting five days. Finally, no one, so far as we are aware, has ever attempted to compare the effects of administration of the two types of nucleic acid by mouth *to the same subjects*. Only a comparison of this kind, obviously, can throw any light upon the relative utilizability of the two types.

With the object of obtaining further information concerning the possibility outlined above, we have sought to ascertain the comparative effects upon the same subjects of oral administration of animal and vegetable nucleic acids in massive doses, superadded to a purine-free diet.

METHODS.

Two experiments were carried out, each of them upon two subjects, C.S.H. and T.B.R. in the one case, and C.S.H. and H.R.M. in the other. The diet consisted of white bread, milk, butter, cheese, eggs, potatoes, and, with the subjects C.S.H. and H.R.M., a little fruit (apple or prunes). Water, wine, and whisky were drunk, but no tea, coffee, cocoa, or beer. In the first experiment, with subjects C.S.H. and T.B.R. no stated quantities were eaten, but appetite in the case of one of us (T.B.R.) was practically uniform throughout the twenty days' duration of the experiment. This was indicated, not only by the amounts eaten, but by the steadiness of the nitrogenous output during the periods when no nucleic acid was being eaten (see charts in figure 1). The other subject (C.S.H.) was somewhat affected by very warm weather which prevailed during the preliminary four-day period of purine-free diet, and as the temperature became more moderate his appetite increased. Nevertheless, a practically uniform protein intake was maintained, an average of four and a half eggs being ingested per diem. The fluctuations of nitrogenous output, which was variable in this subject even during the purine-free period (see charts in figure 2), are to be attributed in the main, therefore, to the temporary condition of the subject, who, as we shall see, suffered a loss of nitrogen throughout the duration of this experiment.

In the second experiment the source of uncertainty constituted by an undefined input of nitrogen was eliminated. Subject C.S.H. varied his foodstuffs slightly, but weighed them daily, and from the weights the nitrogenous input was computed from the standard analyses, tabulated by Lusk (16). Subject H.R.M. ate precisely the same diet daily, after the first twenty-four hours, and the nitrogenous input was therefore constant. The diet of H.R.M. is given below, and so far as constituents and not quantities are concerned it may serve as a description of the diet eaten by the other subjects as well.

Diet Consumed by H.R.M.

First 24 hours.

		Protein.	Fat.	Carbohydrate.	Calories.
Milk	1 litre.	32	38	50	680
Bread	200 grms.	15·1	2·4	105	416
Butter	60 grms.	·6	48·5	—	450
Flour..	100 grms.	8·3	1·0	73·5	254

		Protein.	Fat.	Carbo-hydrate.	Calories.
Eggs ..	190 grms.	24·7	19·0	—	270
Rice ..	100 grms.	2·3	—	77	352
Cheese	60 grms.	12·5	16	12	207
Prunes (Dried)	50 grms.	·8	—	33	135
Orange Juice ..	100 cc.	·6	—	10	46
Estimated Intake		96·9	124·9	360·5	2,810
Whisky, 250 cc.					

Subsequent Days.

Milk ..	1 litre.	32	38	50	680
Bread	380 grms.	26	4·5	198	790
Butter	60 grms.	·6	48·5	—	450
Eggs ..	190 grms.	24·7	19·0	—	270
Rice ..	50 grms.	1·0	—	39	177
Cheese	50 grms.	12·5	16·0	12	207
Prunes or Dried Apricots	50 grms.	·8	—	33	135
	50 grms.	1·8	—	20	125
Orange Juice ..	100 cc.	·6	—	10	46
Estimated Intake		100·0	126·0	362	2,880
Light Wine, 500 cc.					

In each case the entire period of twenty days was divided into five four-day periods. During the first of these nothing was eaten beyond the purine-free diet described above. During the second period the same diet was eaten, with the addition of 15 grms. per day of animal nucleic acid (C.S.H.), or vegetable nucleic acid (T.B.R. and H.R.M.), divided into three doses of 5 grms. each, which were eaten, mixed with a little water, in the morning, at midday, and in the evening respectively. During the third period the diet again consisted of nothing beyond the purine-free materials which have been enumerated. During the fourth period each subject again partook of 15 grms. of nucleic acid per day, but C.S.H. now ate vegetable nucleic acid, while T.B.R. and H.R.M. ate nucleic acid of animal origin. The diet during the fifth and final period against consisted exclusively of purine-free materials. In the case of subject H.R.M. the fifth period was extended to five days.

The vegetable nucleic acid employed in these experiments was derived from yeast and purchased from the British Drug Houses Ltd. Two preparations were used, the one containing 15·70% of nitrogen (theoretical = 16·1%), and 9·17% of phosphorus (theoretical = 9·5%), the other 15·55% of nitrogen and 9·00% of phosphorus. The animal nucleic acid was prepared in this laboratory for the

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[1] The manufacture of the large quantities of animal nucleic acid consumed in this experiment, and others which will form the subject of future communications, was rendered possible by a grant from the Cancer Research Fund of the Commonwealth Department of Health, to whom our thanks and acknowledgments are due.

purposes of this and other experiments by Mr. F. Draper.[1] The source of the nucleic acid was ox-spleen, thymus being not procurable in sufficient amounts. The spleen nucleic acid was prepared by a modification of the method of Levene (14), as follows:

The spleens, in 10-lb. lots, were stripped of their connective tissue covering, ground in a mincer, and transferred to 5 litres of water containing 250 grms. of sodium-hydroxide. The mixture was heated to boiling in enamel buckets, being mechanically stirred meanwhile. Boiling was continued for thirty-five minutes. The mixture was then neutralized with glacial acetic acid, the neutral point, after a little experience, being recognizable by the change of colour of the mixture. Fifty cc. of colloidal iron solution (British Drug Houses Ltd.) were then added, and the mixture filtered. A small amount of $CaCl_2$ solution was added to the filtrate to remove excess of colloidal iron,[2] and the solution again filtered after allowing to stand overnight. To 3 litres of this filtrate were added 500 cc. of concentrated HCl and 8 litres of 98% ethyl alcohol, containing 5% of methyl alcohol. The precipitate was collected and washed with ethyl-methyl alcohol mixture until free from HCl, the final washing being with pure ethyl alcohol. The precipitate was dried at 37°C., and used without further purification. It contained 13·97% of nitrogen (theoretical 14·79%) and 9·23% of phosphorus (theoretical 8·73%).

The urines, collected between 8 a.m. and the same hour on the day following, were preservatized with a solution of thymol in toluene. Total nitrogen was determined by the micro-Kjeldahl method of Folin, urea by the urease method, creatinine colourimetrically with picric acid, phosphates by titration with uranium-acetate (subject T.B.R.) or by the colourimetric method of Briggs (subjects C.S.H. and H.R.M.), chlorides by Volhard's method, ammonia by formol titration, and uric acid by the following modification of the method of Morris and McLeod (17), which has been employed in this laboratory since shortly after the appearance of Morris and McLeod's article.

The reagents required are the following: *Arseno-18-tungstic acid solution.* A mixture of 100 grms. of hydrated sodium tungstate ($Na_2WO_4 2H_2O$), 135 grms. of arsenic acid anhydride (AS_2O_5), and 650 cc. of water is boiled over a small flame for four hours. The reagent is then treated with a sufficient amount of bromine water to render the colour a clear yellow or yellowish brown. After boiling off any excess of bromine, distilled water is added to make the volume of the cooled mixture 1 litre. This reagent yields no colour with phenols.

Standard Uric Acid Solution. Prepared by the method of Benedict and Franke (4) to contain 20 milligrammes of uric acid (Pfansteil) per litre.

Sodium Cyanide Solution. A 3·33% solution of sodium cyanide, to which has been added 1 drop of strong ammonia per litre.

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[2] Otherwise the colloidal iron is carried down by the nucleic acid, and the precipitate is in consequence of a very dark colour and heavily contaminated with iron.

Dilute Hydrochloric Acid. One volume of concentrated acid mixed with nine volumes of water.

The analysis is carried out as follows:

The urine is thoroughly mixed and 1 cc. is withdrawn from the swirling liquid by means of an Ostwald pipette,[3] and introduced into a 100 cc. graduate. Distilled water is added to the 20 cc. mark. One cc. of dilute hydrochloric acid is added to balance the acidity of the uric acid standard.[4] Ten cc. of sodium cyanide are added, followed by 2 cc. of the arseno-tungstic acid reagent. The contents of the graduate are mixed, and at the end of five minutes are diluted to the 100 cc. mark and again mixed. The blue solution is compared in a colourimeter with a simultaneously prepared solution, obtained by treating 20 cc. of the standard uric acid solution (containing 0·4 mg. of uric acid) in a 100 cc. graduate, with 10 cc. of the sodium cyanide solution and 2 cc. of the arseno-tungstic acid reagent, diluting the mixture to the 100 cc. mark at the end of five minutes.

The faeces which were passed in each day were evaporated nearly to dryness *in vacuo* over a boiling water bath, drying being completed by heating the residue in an open dish on the boiling water bath. The residues were weighed and then coarsely pulverized. Five gramme samples from each day's passage were ignited for several hours, ignition being aided in the cases of subjects T.B.R. and H.R.M. by addition of a few drops of strong nitric acid. The ash was almost completely soluble in 5 cc. of hot concentrated hydrochloric acid. The very small residue was re-extracted with a few drops of hot concentrated hydrochloric acid. The solution, washing and residue of silicates, were introduced into a 100 cc. flask, and when cool diluted to the 100 cc. mark. The phosphoric acid was determined in 1 cc. of this solution by the colourimetric method of Briggs, using for comparison a standard containing either 0·5 or 1·0 mg. of phosphorus.

In the second experiment upon subject C.S.H. the concentration of uric acid in the blood was determined on the last day of each of the four-day periods. Five cc. of blood was withdrawn from the median basilic vein and deproteinized by the method of Folin. The uric acid in the protein-free filtrates was determined by the method of Benedict (2).[5]

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[3] In many of the urines passed during the periods of vegetable nucleic acid ingestion, the crystals of uric acid were so large and numerous as to render very questionable the accuracy of sampling by this method. In such cases one of two procedures was adopted. Either a measured quantity of normal NaOH was added to the whole of the urine until the greater part of the uric acid was dissolved, allowance being subsequently made for the alteration of volume and titratable acidity thus brought about, or else a large aliquot was subtracted from the whole volume, and to this 10% NaOH was added until the uric acid was dissolved, allowance being made in the analysis for the dilution of the urine by the sodium hydroxide which was added.

[4] This is essential because the colour which subsequently develops varies with the reaction of the mixture.

[5] We desire to express our indebtedness to Mr. F. Draper for his kindness in carrying out these blood analyses for us.

TABLE 1. SUBJECT T.B.R.

	Urine.									Faeces.		
Date	Volume cc.	Titratable Acidity in cc. tenth normal alkali per day	Ammonia Grammes	Urea + Ammonia Nitrogen Grammes	Total Nitrogen Grammes	Creatinine Grammes	Uric Acid Grammes	P_2O_5 Grammes	Chlorides as NaCl Grammes	Total Weight	P_2O_5 per cent.	Total P_2O_5 Grammes
Purine-free Period.												
29.XI.26	790	395	0·56	11·06	12·85	1·81	0·41	2·05	Not determined	78·6	3·87	3·04
30.XI.26	700	434	0·57	10·50	12·61	2·08	0·38	2·19	Not determined	None	—	—
1.XII.26	745	462	0·60	10·88	13·80	2·12	0·36	2·28	Not determined	23·9	5·63	1·35
2.XII.26	820	435	0·63	11·63	13·33	2·02	0·40	2·57	Not determined	None	—	—
Vegetable Nucleic Acid Period.												
3.XII.26	1,230	689	0·84	14·39	15·97	2·32	0·91	3·75	Not determined	19·94	8·43	1·68
4.XII.26	1,230	738	0·98	12·94	15·50	2·20	1·05	3·91	Not determined	None	—	—
5.XII.26	1,510	695	1·10	12·74	14·66	2·39	1·10	3·96	11·3	43·5	9·89	4·31
6.XII.26	1,870	767	1·59	13·31	15·20	2·43	1·31	4·59	13·9	None	—	—
Purine-free Period.												
7.XII.26	920	644	1·19	10·95	13·05	2·26	0·50	3·56	9·5	41·4	7·19	2·97
8.XII.26	1,060	551	0·92	11·78	12·93	2·14	0·49	2·80	14·2	10·3	4·98	0·51
9.XII.26	880	484	0·81	10·30	11·73	2·09	0·35	2·45	11·3	None	—	—
10.XII.26	700	476	0·73	9·93	11·38	2·02	0·29	2·29	7·0	8·5	5·26	0·45
Animal Nucleic Acid Period.												
11.XII.26	1,090	600	0·93	13·30	14·63	2·43	0·50	3·17	9·1	85·8	5·78	4·96
12.XII.26	970	601	1·06	12·05	13·75	2·32	0·49	3·42	8·7	None	—	—
13.XII.26	1,470	617	1·10	12·86	14·77	2·21	0·56	3·50	16·5	19·18	9·71	1·86
14.XII.26	1,200	660	1.16	13·04	14·63	2·18	0·61	3·72	11·3	None	—	—
Purine-free Period.												
15.XII.26	1,020	551	1·02	11·79	13·86	2·26	0·49	3·02	13·3	None	—	—
16.XII.26	790	490	0·87	11·12	12·25	2·18	0·49	2·45	10·1	36·2	8·94	3·23
17.XII.26	630	447	0·80	11·15	12·11	1·96	0·37	2·19	6·8	11·1	8·43	0·94
18.XII.26	780	460	0·72	12·68	14·31	2·14	0·46	2·49	8·7	None	—	—

EFFECTS OF THE ADMINISTRATION OF NUCLEIC ACIDS UPON EXCRETION.

Considering in the first place the results obtained with subject T.B.R., it is evident from the charts (figure 1) that the volume of urine and its titratable acidity, ammonia, phosphate, uric acid, urea, and total nitrogen contents were all increased during the experimental periods, but very much more during the vegetable nucleic acid period than during the period when animal nucleic acid was partaken. The chlorides merely follow the urinary volume. The discrepancy between the two periods is particularly marked in the case of the uric acid output, which rose to 0·65, 0·70, and 0·91 grms. above the endogenous output during the last three days of the vegetable nucleic acid period, and only attained 0·21 grms. in excess of the endogenous output in the last day of the animal nucleic acid period. The increase of urinary phosphate output during the vegetable nucleic acid period was similarly nearly double the increase which was observed during the period of partaking animal nucleic acid (Table 2).

TABLE 2. SUBJECT T.B.R.

Excess Excretion following Ingestion of Nucleic Acid.

	Vegetable Nucleic Acid.				Animal Nucleic Acid.			
	Nitrogen.	P_2O_5.		Uric Acid.	Nitrogen.	P_2O_5.		Uric Acid.
Day.		Urine.	Faeces.			Urine.	Faeces.	
1st day of Ingestion ..	3·12	1·25	0·68	0·51	1·78	0·67	0·67	0·10
2nd ,, ,,	2·65	1·41	—	0·65	0·90	0·92	—	0·09
3rd ,, ,,	1·81	1·46	2·12	0·70	1·92	1·00	0·90	0·16
4th ,, ,,	2·35	2·09	—	0·91	1·78	1·22	—	0·21
1st day of Post-period	0·20	1·06	0·91	0·10	1·01	0·52	—	0·09
2nd ,, ,,	0·08	0·30	0·00	0·09	0·00	0·00	1·43	0·09
3rd ,, ,,	0·00	0·00	—	0·00	0·00	0·00	0·38	0·00
4th ,, ,,	0·00	0·00	0·02	0·00	(1·46)	0·00	—	0·06
Totals	10·21	7·57	3·73	2·96	7·39	4·33	3·38	0·80
		11·30			(8·85)	7·71		
Ingested	9·42	12·60		15·10	8·38	12·68		13·70
Balance*	—0·79	+1·30		+12·14	+0·99 (—0·47)	+4·97		+12·90

*A + sign indicates retention by the subject, a — sign indicates loss.

So far as the other metabolites enumerated in the following tables are concerned, the basal output is computed as the average of the outputs on the four days of the fore-period, except in the case of uric acid in which the higher output of the first day, representing delayed excretion consequent upon previous ingestion of purines, is rejected. In the case of the faecal phosphate output of this

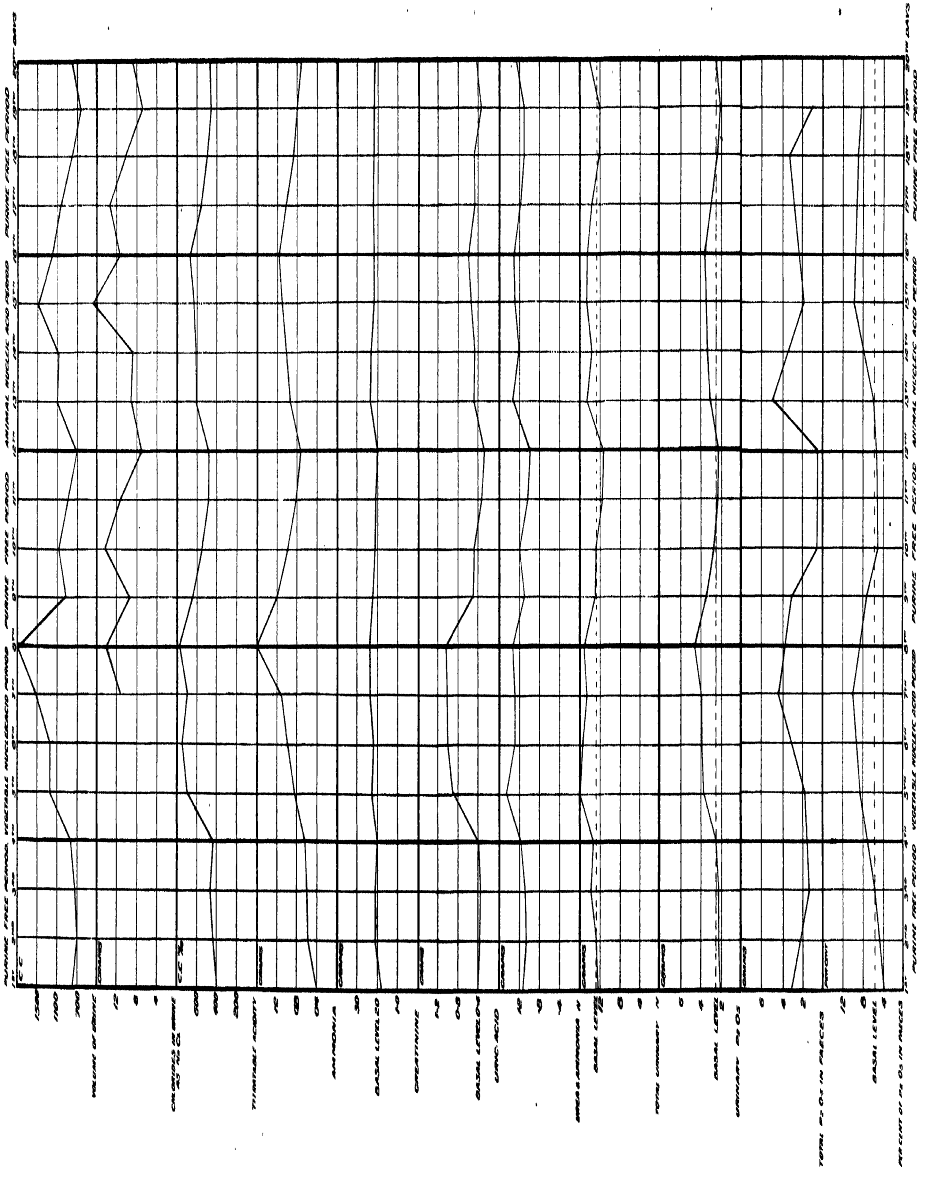

Figure 1. Subject T.B.R.

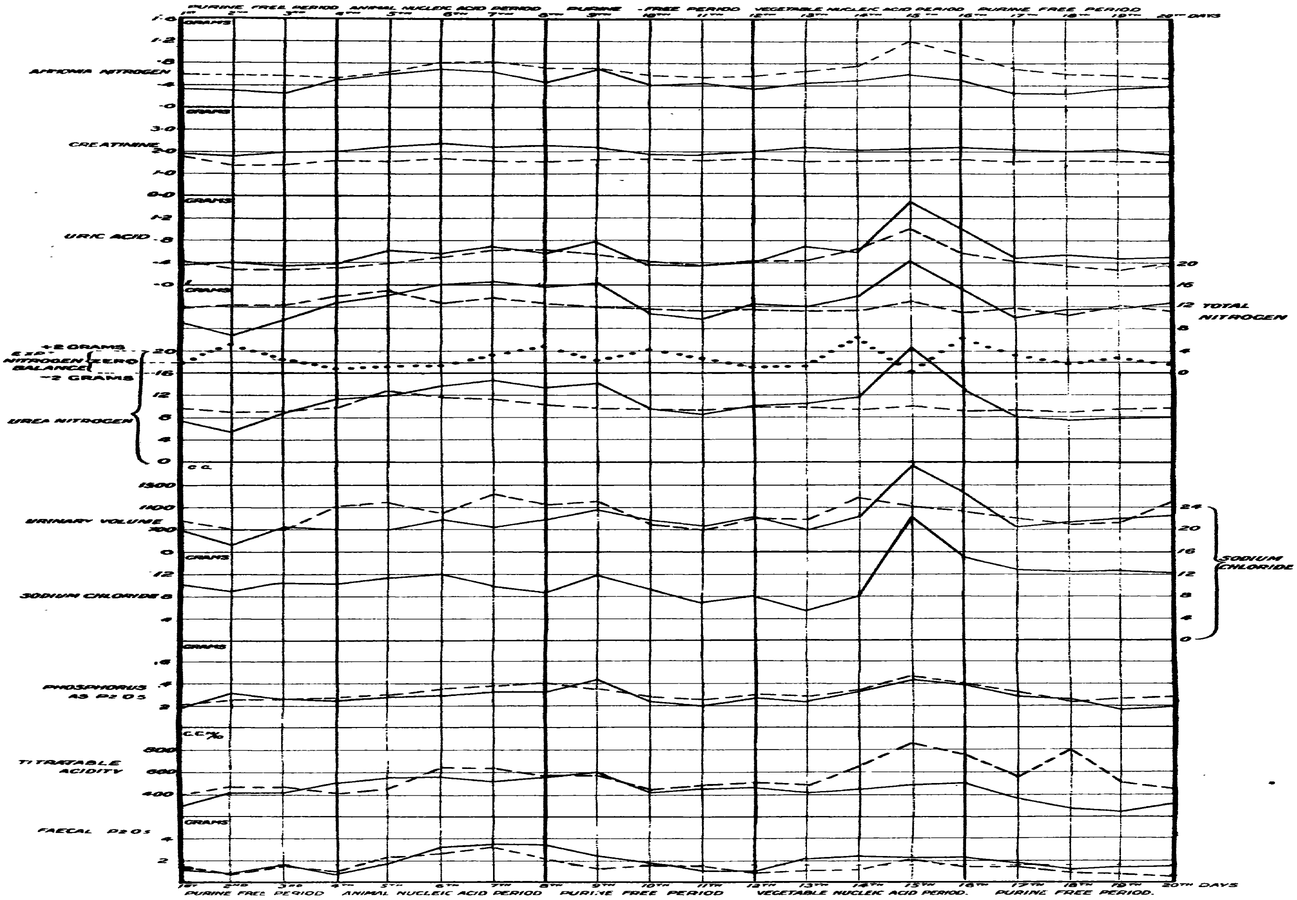

Figure 2. Subject C.S.H.

Continuous line: Experiment 1.
Broken line: Experiment 2.

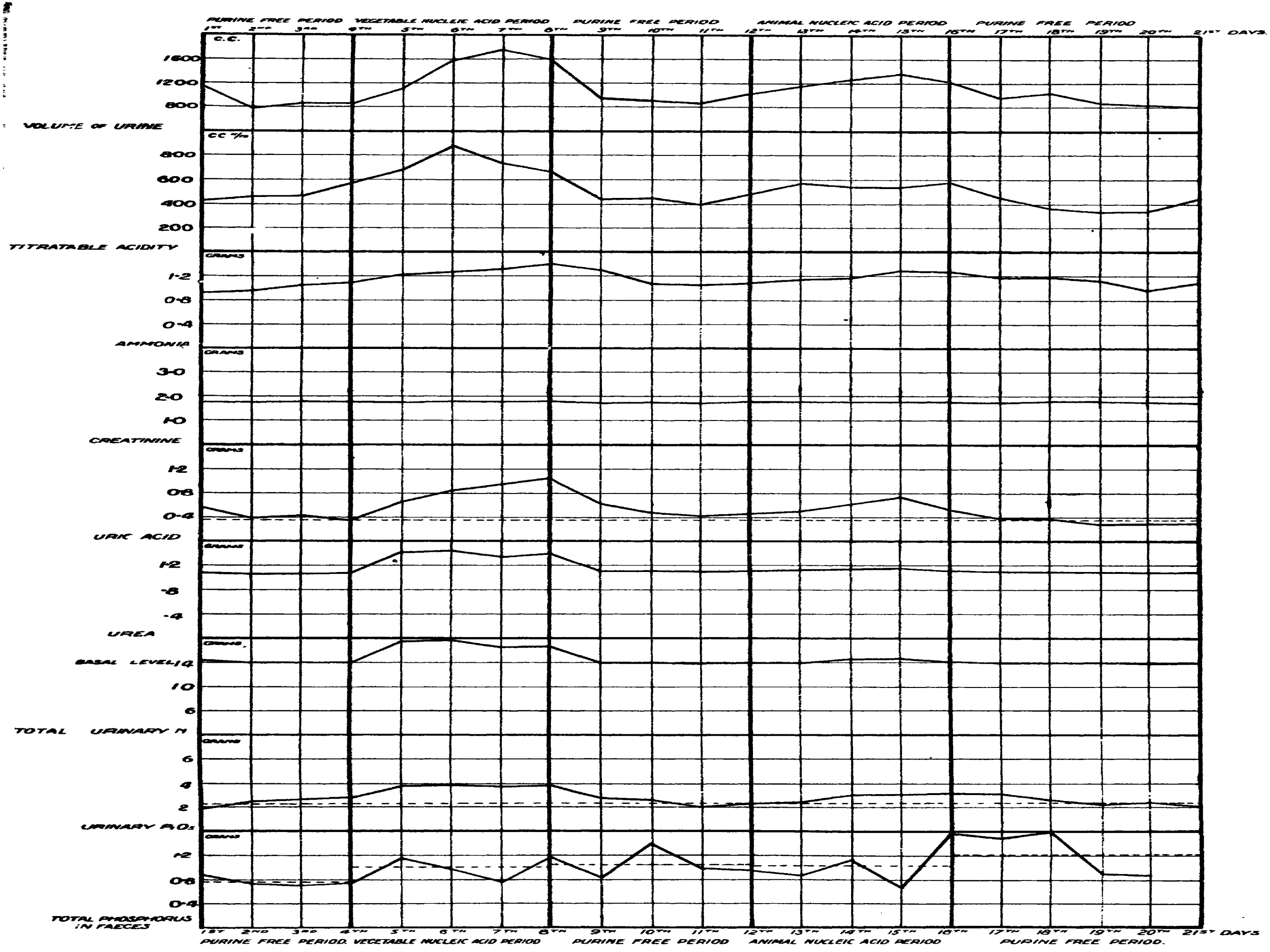

Figure 3. Subject H.R.M.

subject, however, considerable difficulty is created by the irregularity with which faeces were passed, an irregularity which was attributable in large measure to the nature of the diet. In consequence of the frequent retention of faeces for a day, the *percentage* of phosphates in the faeces probably presents a fairer index of excess excretion than the absolute amount passed on any day, since the latter figure varied with the duration of retention and completeness of evacuation. 5% of P_2O_5 was assumed to represent the basal level of faecal phosphate output, since this was the lowest percentage of P_2O_5 in any sample of faeces passed (on the third day of the first post-period, corresponding to food partaken on the second day of the post-period, see Table 1). On this basis the excess faecal phosphate output was about the same for both types of nucleic acid. The absorption of the two types of nucleic acid from the alimentary canal was therefore about equal.

In the case of the vegetable nucleic acid, the whole of the nitrogenous input was somewhat more than accounted for in the urinary output during the period of ingestion and the succeeding four days, so that there was a negative nitrogen balance. In the post-period following upon the animal nucleic acid period there was a sudden rise of nitrogenous output in the fourth day, following upon two days of subnormal output. The origin of this rise is uncertain. It may represent fluctuation of appetite, or it may represent a sudden release of stored-up products of nucleic acid metabolism, but it is significant that if the defects of excretion during the preceding two days are added together they total 1·34 grms., which is almost exactly the excess encountered on the succeeding day. Possibly, therefore, this nitrogen was not derived from the ingested nucleic acid, in which case only 88% of the ingested nitrogen is accounted for. If the excess nitrogen excreted on the fourth day of the post-period be regarded as derived from the nucleic acid ingested four or more days previously, however, then the whole, and somewhat more than the whole, of the ingested nitrogen is accounted for, as in the case of the vegetable nucleic acid, but the delayed excretion represents a much larger proportion of the total, and the negative balance is smaller, that is, retention is greater in the case of animal than in the case of vegetable nucleic acid.

The urinary excretion of P_2O_5 corresponded to 60% of that ingested in the case of vegetable nucleic acid and only 34% of that ingested in the case of animal nucleic acid. Adding the faecal excretion of phosphates, the total output was 90% of that ingested in the case of vegetable nucleic acid, only 61% in the case of animal nucleic acid. There was, therefore, a very considerable retention of phosphoric acid following upon ingestion of animal nucleic acid, and as in the case of nitrogen the retention of P_2O_5 was greater with animal than with vegetable nucleic acid.

The difference in the behaviour of the two types of nucleic acid, following upon their ingestion, however, is more strikingly evident in the comparative outputs of uric acid than in any other particular, for while ingestion of a total of 60 grms. of vegetable nucleic acid led to the excretion of an excess of 2·96 grms. of uric acid, or 20% of the theoretical yield from the purine bases of the nucleic acid, ingestion of the same amount of animal nucleic acid led to excess excretion of only 0·80 grms. of uric acid, or 5·8% of the theoretical yield.

In the case of C.S.H. (first experiment) there was some difficulty in assigning a basis for estimation of the nitrogen balance, because this subject started with a very low nitrogenous intake, which was subsequently amended. In

TABLE 3. SUBJECT C.S.H. EXPERIMENT 1.

Date	Volume cc.	Ammonia Nitrogen Grammes	Urea Nitrogen Grammes	Total Nitrogen Grammes	Creatinine Grammes	Uric Acid Grammes	Chlorides as NaCl Grammes	P_2O_5 Grammes	Faecal P_2O_5 Grammes
Purine-free Period.									
30.XI.26	680	0·36	7·55	9·06	1·90	0·36	3·10	1·95	1·15
1.XII.26	550	0·32	5·55	6·80	1·80	0·43	4·27	3·23	1·04
2.XII.26	720	0·24	8·72	9·60	2·08	0·35	4·34	2·76	1·58
3.XII.26	700	0·53	11·20	12·73	2·02	0·36	3·86	2·53	1·33
Animal Nucleic Acid Period.									
4.XII.26	700	0·66	12·10	14·00	2·25	0·63	4·60	2·87	1·73
5.XII.26	825	0·69	13·60	16·13	2·37	0·56	6·11	2·99	3·12
6.XII.26	710	0·63	14·80	16·50	2·20	0·68	6·86	3·36	3·50
7.XII.26	870	0·45	13·30	15·80	2·25	0·56	6·78	3·33	3·45
Purine-free Period.									
8.XII.26	1,060	0·70	14·13	16·30	2·20	0·77	6·74	4·37	2·37
9.XII.26	770	0·42	9·62	10·62	1·90	0·38	4·17	2·40	1·77
10.XII.26	730	0·36	8·53	9·73	1·90	0·36	3·10	2·07	1·03
11.XII.26	920	0·46	10·10	12·60	2·00	0·40	3·95	2·88	1·07
Vegetable Nucleic Acid Period.									
12.XII.26	700	0·46	10·57	12·17	2·40	0·70	4·56	2·40	2·16
13.XII.26	910	0·48	11·70	14·00	2·16	0·60	7·00	3·33	3·67
14.XII.26	1,860	0·62	18·60	20·70	2·15	1·48	7·53	4·28	2·25
15.XII.26	1,365	0·59	12·40	14·80	2·21	1·09	6·21	3·91	2·30
Purine-free Period.									
16.XII.26	750	0·25	8·10	10·00	2·10	0·49	4·71	2·87	1·84
17.XII.26	820	0·25	9·65	11·30	2·00	0·52	3·99	2·53	1·36
18.XII.26	880	0·32	9·77	11·70	2·20	0·46	3·24	1·73	1·51
19.XII.26	900	0·37	10·60	12·50	1·87	0·48	3·38	2·00	1·38

consequence of this the nitrogenous intake and output rose during the last three days of the preliminary purine-free period (see charts in figure 2 and Tables 3 and 4). By the fourth day of the pre-period, however, when the subject had become accustomed to the diet, the intake had reached a steady level, and the output corresponding closely with that attained on the fourth days of both post-periods. The basal output of nitrogen on the purine-free diet was therefore estimated to be 12·5 grms. per diem, and, as it happened when the experiment was subsequently repeated upon this subject, this figure also represented exactly

TABLE 4. SUBJECT C.S.H. EXPERIMENT 2.

Date	Volume cc.	Titratable Acidity in cc. tenth normal alkali per day	Ammonia Grammes	Urea + Ammonia Nitrogen Grammes	Total Nitrogen Grammes	Creatinine Grammes	Uric Acid Grammes	P_2O_5 Grammes	Faecal P_2O_5 Grammes
Purine-free Period.									
25.IV.27	850	408	0·74	10·4	11·9	1·57	0·430	2·13	1·39
26.IV.27	695	480	0·71	10·6	12·2	1·40	0·282	2·59	0·70
27.IV.27	695	480	0·71	10·6	12·2	1·40	0·282	2·59	1·65
28.IV.27	1,110	422	0·66	11·4	13·5	1·60	0·317	2·67	0·65
Animal Nucleic Acid Period.									
29.IV.27	1,670	468	0·77	13·5	14·6	1·56	0·391	3·06	2·18
30.IV.27	970	653	1·02	10·7	12·6	1·70	0·498	3·58	2·65
1.V.27	1,310	647	1·01	12·2	13·5	1·62	0·623	3·84	3·31
2.V.27	1,120	582	0·88	11·3	12·6	1·53	0·623	3·33	2·06
Purine-free Period.									
3.V.27	1,170	575	0·88	10·6	12·0	1·64	0·548	3·50	1·02
4.V.27	780	437	0·73	10·1	11·7	1·68	0·434	2·84	1·52
5.V.27	680	449	0·69	9·9	11·6	1·60	0·340	2·66	1·58
6.V.27	895	510	0·72	10·5	11·7	1·68	0·428	3·07	0·97
Vegetable Nucleic Acid Period.									
7.V.27	880	484	0·81	10·6	11·6	1·55	0·429	2·89	1·08
8.V.27	1,270	664	0·93	10·2	11·4	1·56	0·650	3·41	1·10
9.V.27	1,110	863	1·48	11·3	12·8	1·60	1·041	4·73	2·04
10.V.27	1,000	763	1·05	10·1	11·4	1·65	0·631	3·98	1·50
Purine-free Period.									
11.V.27	890	561	0·86	10·1	11·8	1·58	0·427	3·31	0·80
12.V.27	770	468	0·73	9·7	10·9	1·60	0·342	2·46	0·83
13.V.27	820	500	0·71	10·2	12·0	1·55	0·262	2·70	0·74
14.V.27	775	448	0·63	10·2	11·7	1·65	0·236	2·82	0·58

the average of the outputs on the four days of the pre-period in that experiment. Since the intake in this latter experiment was regulated by appetite, and the initial fluctuations in the first experiment represented progressive adjustments to appetite, we may with some confidence assume that this estimate of the basal output is approximately correct.

Whether we assume the basal output of nitrogen to have been 12·5 grms. or less, however, it remains evident that in this subject a negative nitrogen balance was maintained throughout the duration of the experiment. On the assumed basis the loss of nitrogen during the animal nucleic acid period and the post-period which followed it was 7·95 grms., while during the plant nucleic acid period and the post-period which followed the loss was 2·58 grms. If we take the basal output at the lower figure indicated by the average of the pre-period outputs these losses would be much greater, but the loss during the vegetable nucleic acid period would, of course, continue to be less than the loss during the animal nucleic acid period. The relationship of nitrogen output to the type of nucleic acid ingested was therefore the opposite of that observed in subject T.B.R.

With the subject C.S.H. the regularity of evacuation permitted a steady observation of the faecal phosphate output which was not possible in the subject T.B.R. The excess output of urinary phosphates amounted to 32% of the input in the case of animal nucleic acid, and 31% in the case of vegetable nucleic acid. There was not that disproportion, therefore, between the urinary phosphate outputs with the two types of nucleic acid which was observed in subject T.B.R. The total excess output, urinary and faecal, was 97% of the intake in the case of animal nucleic acid, and 80% in the case of vegetable nucleic acid. With the phosphates, as with the nitrogen, therefore, the results are the opposite of those obtained with subject T.B.R., retention being greater with vegetable than with animal nucleic acid.

When, however, we consider the uric acid excretion the two cases are parallel, and substantially the same results are obtained. Much more uric acid was excreted during the vegetable nucleic acid period and subsequent post-peroid than during the animal nucleic acid period and subsequent post-period. The total excess output arising from vegetable nucleic acid was 18% of the theoretical yield, while that arising from animal nucleic acid was only 9·6% of the theoretical yield.

A totally unanticipated result of the ingestion of both kinds of nucleic acid by these subjects was an increase, slight but very definite, of the apparent output of creatinine (see figures 1 and 2). This occurred in both subjects, and in both the characteristics of the effect were the same. The creatinine output rose in the first twenty-four hours to a value about 15% above the basal output. It stayed at this level throughout the period of ingestion, and then slowly sank, reattaining basal value towards the end of the succeeding purine-free period.

The maximum output of creatinine was somewhat less with animal than with vegetable nucleic acid, and it regained the basal value more quickly during the following period of purine-free diet.

For estimating the creatinine, however, picric acid was employed which had been repeatedly recrystallized from water, but not from benzene. In the subsequent experiments, about to be described, the picric acid which was employed for the creatinine estimations had been repeatedly recrystallized from benzene (3). With this picric acid no rise of creatinine output consequent upon nucleic acid ingestion could be observed. On the contrary, while C.S.H. shows a slight irregular vacillation of creatinine output (figure 2), the other subject of the second experiment, H.R.M., illustrates the classical uniformity of excretion of this metabolite (figure 3). We must conclude, therefore, that the ingestion of nucleic acid leads to an increased output of those substances of unknown nature which react with incompletely purified picric acid, and that the apparent rise of creatinine values during the nucleic acid periods in the first two experiments was entirely attributable to these substances.

The contradictory results in respect to nitrogen and phosphate output with the first two subjects led to a repetition of the experiment. It appeared possible that subject C.S.H. was in an abnormal condition during the first experiment, since he suffered a loss of nitrogen throughout the duration of the experiment, and experienced subjective symptoms of distress which will be described more fully later on. The experiment was therefore repeated upon that subject nearly five months later, and also upon another subject, H.R.M.

In the second experiment with C.S.H. all food consumed was weighed, but freedom of relative choice was maintained. The nitrogen intake was therefore variable, but the relative values could be computed from standard tables. These estimated intakes of nitrogen on each day of the experiment are given in the accompanying table; such estimates are, of course, to be taken as relative only. The "nitrogen balance" depicted upon the graphs in figure 2 represents the difference between these estimates of intake and the actual output, reckoned as positive when the estimated intake exceeded the output.

C.S.H., SECOND EXPERIMENT.

Estimated Nitrogen Intake in Grammes.

	Pre-period.	Animal Nucleic Acid Period.	Post-period.	Vegetable Nucleic Acid-Period.	Post-period.
First day ..	14·1	13·8	12·4	11·8	12·9
Second day ..	15·5	13·2	14·0	13·8	14·4
Third day ..	13·3	12·3	12·1	10·8	13·3
Fourth day..	12·4	15·4	10·6	15·2	12·3
Averages ..	13·8	13·7	12·3	12·9	13·2

Contrary to the previous experiment, the nitrogen balance (estimated from the basal output during the four days of the pre-period) was positive throughout the duration of the experiment. The retention of nitrogen, however, as before, was greater during the second vegetable nucleic acid period than during the first animal nucleic acid period. The urinary excess output of phosphates amounted to 46% of the intake in the animal nucleic acid period and post-period, and 51% of the intake in the vegetable nucleic acid period and post-period. The faecal phosphate output, contrary to the first experiment, did not greatly exceed the urinary output in the animal nucleic acid period, and was much less than the urinary output in the vegetable nucleic acid period. Assimilation of the ingested nucleic acid was therefore much more complete in the second experiment than in the first. The total output of phosphates was 99% of the intake for the animal nucleic acid and 62% of the intake for the vegetable nucleic acid. As in the first experiment, therefore, the retention of phosphorus was greater during the period of vegetable nucleic acid ingestion than it was during the animal nucleic acid period.

As before, the uric acid excretion during and following the vegetable nucleic acid period exceeded that obtained in consequence of the ingestion of animal nucleic acid, but not to so great an extent as in the first experiment, the excess excretion of uric acid in the vegetable nucleic acid period and post-period being 11·7% of theoretical, while the excess excreted during the animal nucleic acid period and post-period was 11·1% of theoretical.

In the experiment with H.R.M. all food was weighed, and the intake was kept rigorously constant. The diet eaten by this subject has been set forth in detail in the introductory portion of this article. The uniformity of nitrogenous intake was reflected in the constancy of the basal nitrogenous output (Table 4).

The nitrogen balance was negative during the first or vegetable nucleic acid period and post-period, and positive during the second or animal nucleic acid period and post-period. As with subject T.B.R., therefore, retention was greater in the animal nucleic acid period and post-period than in the vegetable nucleic acid period and post-period.

The excess urinary output of phosphates constituted 69% of the intake in the case of vegetable nucleic acid, and 46% of the intake in the case of animal nucleic acid. The excess faecal phosphates were in both cases considerably less in amount than the excess urinary phosphates, but were greater in amount during the animal nucleic acid period and post-period than during the vegetable nucleic acid period and post-period. The absorption of animal nucleic acid and its products from the alimentary canal may therefore have been somewhat less complete with the animal than with the vegetable nucleic acid. The total excess output of phosphates was 89% of the intake with vegetable nucleic acid, and 72% of the intake with animal nucleic acid. In each case, therefore, retention occurred, but, as in the previous experiment with T.B.R., the retention was greater with animal than with vegetable nucleic acid.

TABLE 5. SUBJECT C.S.H.

Excess Excretion following Ingestion of Nucleic Acid.

	Animal Nucleic Acid.				Vegetable Nucleic Acid.			
	Nitrogen.	P₂O₅.		Uric Acid.	Nitrogen.	P₂O₅.		Uric Acid.
Day.		Urine.	Faeces.			Urine.	Faeces.	
First Experiment.								
1st day of Ingestion ..	1·50	0·25	0·45	0·25	0·00	0·00	0·88	0·32
2nd ,, ,,	3·63	0·37	1·84	0·18	1·50	0·71	2·39	0·22
3rd ,, ,,	4·00	0·74	2·22	0·30	8·20	1·66	0·97	1·10
4th ,, ,,	3·30	0·71	2·17	0·18	2·30	1·29	1·02	0·71
1st day of Post-period	3·80	1·75	1·09	0·39	0·00	0·25	0·56	0·11
2nd ,, ,,	0·00	0·00	0·49	0·00	0·00	0·00	0·08	0·14
3rd ,, ,,	0·00	0·00	0·00	0·00	0·00	0·00	0·23	0·08
4th ,, ,,	0·10	0·26	0·00	0·02	0·00	0·00	0·10	0·10
Totals	16·33	4·08	8·26	1·32	12·00	3·91	6·23	2·78
		12·34				10·14		
Ingested	8·38	12·68		13·70	9·42	12·60		15·10
Balance*	—7·95	+0·34		12·38	—2·58	+2·46		+12·32
Second Experiment.								
1st day of Ingestion ..	2·1	0·56	1·08	0·10	0·00	0·39	0·00	0·14
2nd ,, ,,	0·1	1·08	1·55	0·20	0·00	0·91	0·00	0·36
3rd ,, ,,	1·0	1·34	2·21	0·33	0·30	2·23	0·94	0·75
4th ,, ,,	0·1	0·83	0·96	0·33	0·00	1·48	0·40	0·34
1st day of Post-period	0·0	1·00	0·00	0·25	0·00	0·81	0·00	0·13
2nd ,, ,,	0·0	0·34	0·42	0·14	0·00	0·00	0·00	0·05
3rd ,, ,,	0·0	0·16	0·48	0·05	0·00	0·20	0·00	0·000
4th ,, ,,	0·0	0·57	0·00	0·13	9·00	0·32	0·00	0·000
Totals	3·3	5·88	6·70	1·53	0·30	6·34	1·34	1·770
		12·58				7·68		
Ingested	8·4	12·68		13·70	9·3	12·37		15·10
Balance*	+5·1	+0·10		+12·17	+9·0	+4·69		+13·33

*A + sign indicates retention by the subject, a — sign indicates loss.

As in the other experiments, the output of uric acid was much higher in this subject when vegetable nucleic acid was ingested than when animal nucleic acid was ingested. The excess excretion of uric acid during and following the vegetable nucleic acid period was 18% of theoretical, while during and following the animal nucleic acid period it was only 7·4% of theoretical.

TABLE 6. SUBJECT H.R.M.

Date	Volume cc.	Titratable Acidity in cc. tenth normal alkali per day	Ammonia Grammes	Urea + Ammonia Nitrogen Grammes	Total Nitrogen Grammes	Creatinine Grammes	Uric Acid Grammes	P_2O_5 Grammes	Faecal P_2O_5 Grammes
Purine-free Period.									
25.IV.27	1,150	426	0·92	11·8	14·3	1·77	0·565	1·82	0·886
26.IV.27	775	458	0·95	11·5	13·9	1·75	0·380	2·40	0·734
27.IV.27	850	459	1·06	11·5	13·9	1·79	0·420	2·59.	0·701
28.IV.27	840	572	1·10	11·6	13·8	1·77	0·344	2·73	0·724
Vegetable Nucleic Acid Period.									
29.IV.27	1,085	685	1·21	15·1	17·4	1·74	0·639	3·71	1·115
30.IV.27	1,560	884	1·27	15·7	17·6	1·75	0·815	3·73	0·955
1.V.27	1,670	741	1·32	14·8	16·4	1·77	0·931	3·65	0·749
2.V.27	1,580	668	1·40	14·9	16·8	1·76	1·121	3·76	1·172
Purine-free Period.									
3.V.27	940	440	1·29	12·1	13·9	1·74	0·611	2·76	0·823
4.V.27	900	450	1·09	12·3	13·9	1·75	0·471	2·58	1·384
5.V.27	840	395	1·06	12·4	13·8	1·74	0·417	2·00	0·967
6.V.27	1,000	490	1·11	11·8	13·9	1·76	0·449	2·30	0·962
Animal Nucleic Acid Period.									
7.V.27	1,120	575	1·15	12·0	13·8	1·76	0·497	2·45	0·847
8.V.27	1,235	543	1·18	12·2	13·8	1·76	0·603	3·06	1·086
9.V.27	1,320	541	1·30	12·4	14·3	1·74	0·734	3·05	0·648
10.V.27	1,200	576	1·27	12·4	13·8	1·74	0·508	3·14	1·520
Purine-free Period.									
11.V.27	930	451	1·18	11·9	13·7	1·74	0·372	3·05	1·464
12.V.27	1,000	480	1·19	11·8	13·8	1·76	0·364	2·57	1·580
13.V.27	840	437	1·14	11·9	13·4	1·76	0·282	2·16	0·880
14.V.27	800	464	0·94	11·7	13·7	1·76	0·281	2·36	0·850
15.V.27	790	550	1·09	11·8	13·9	1·74	0·282	2·02	—

To sum up the effects of nucleic acids upon excretion, therefore, it is evident that in subjects T.B.R. and H.R.M., who partook of vegetable nucleic acid first and animal nucleic acid later, retention of nitrogen and phosphorus, during the period occupied by the experiment, was greater with animal nucleic acid than when the administered nucleic acid had been derived from yeast, while in two experiments upon subject C.S.H., who partook of animal nucleic acid first and

TABLE 7. SUBJECT H.R.M.

EXCESS EXCRETION FOLLOWING INGESTION OF NUCLEIC ACID.

	Vegetable Nucleic Acid.				Animal Nucleic Acid.			
	Nitrogen.	P_2O_5.		Uric Acid.	Nitrogen.	P_2O_5.		Uric Acid.
Day.		Urine.	Faeces.			Urine.	Faeces.	
1st day of Ingestion ..	3·6	1·71	0·415	0·295	0·0	0·45	0·147	0·153
2nd „ „	3·8	1·73	0·255	0·471	0·0	1·06	0·386	0·259
3rd „ „	2·6	1·65	0·049	0·587	0·5	1·05	0·000	0·390
4th „ „	3·0	1·76	0·472	0·777	0·0	1·14	0·820	0·164
1st day of Post-period	0·1	0·76	0·123	0·267	0·0	1·05	0·764	0·028
2nd „ „	0·1	0·58	0·684	0·127	0·0	0·57	0·880	0·020
3rd „ „	0·0	0·00	0·267	0·073	0·0	0·16	0·180	0·000
4th „ „	0·1	0·30	2·262	0·105	0·0	0·36	0·150	0·000
5th „ „	—	—	—	—	0·0	0·02	—	0·000
Totals	13·3	8·49	2·527	2·702	0·5	5·86	3·327	1·014
		11·02				9·19		
Ingested	9·3	12·37		15·10	8·4	12·68		13·70
Balance*	—4·0	+1·35		+12·40	+7·9	+3·49		+12·69

*A + sign indicates retention by the subject, a — sign indicates loss.

vegetable nucleic acid later, retention of nitrogen and phosphorus was greater with vegetable than with animal nucleic acid. In three experiments out of four the faecal phosphates formed a greater proportion of the total when animal nucleic acid was eaten than when vegetable nucleic acid was eaten, indicating somewhat less complete absorption of animal nucleic acid from the alimentary canal. In the case of subject T.B.R. absorption was approximately the same for both types of nucleic acid. The following table shows the distribution of phosphorus between the urine and faeces in the various experiments:

TABLE 8.

Distribution of Phosphorus between Urine and Faeces.

		Percentage of Ingested Phosphorus excreted in			
		Urine.		Faeces.	
Subject.	Order in which Nucleic Acids were administered.	Vegetable Nucleic Acid.	Animal Nucleic Acid.	Vegetable Nucleic Acid.	Animal Nucleic Acid.
T.B.R.	Animal following Vegetable	60	34	30	27
H.R.M.	Animal following Vegetable	69	46	20	26
C.S.H.1.	Vegetable following Animal	31	32	49	65
C.S.H.2.	Vegetable following Animal	51	46	11	53

In every case the excess excretion of uric acid following upon ingestion of nucleic acid was far less than might theoretically have been derived from the purine bases in the nucleic acid, and inspection of the charts shows quite clearly that the remainder of the purine-nitrogen was largely accounted for by an increased output of urea. In every case uric acid excretion was greater with vegetable nucleic acid than with nucleic acid of animal origin. The volume of urine, titratable acidity, and ammonia output were all greater with vegetable than with animal nucleic acid.

OTHER EFFECTS FOLLOWING INGESTION OF LARGE QUANTITIES OF NUCLEIC ACID.

Two subjects (T.B.R. and H.R.M.) experienced a subjective feeling which is best described as one of "strain" or "nervous tension" during the period of vegetable nucleic acid ingestion. The effect was no greater than would be amply accounted for by the slight degree of acidosis which was undoubtedly present, and which culminated on the fourth day, when the subjective symptoms also attained their maximum intensity. During the period of animal nucleic acid ingestion no subjective symptoms or discomfort of any kind were experienced by these subjects.

In the case of C.S.H. the ingestion of 15 grms. of animal nucleic acid per diem was followed by a steady increase of feelings of personal discomfort. In the first experiment this was attributed in part to excessive perspiration in hot weather, to which this subject was unaccustomed, causing corresponding concentration of his urine. Excess of distilled water was drunk, but without any objective result or easing of symptoms until the ninth day (see charts in figure 2), when a sudden rise in excretion was followed by some relief of symptoms. There would seem to have been some delay of excretion.

During the post-period the symptoms of depression abated, and did not recur with the onset of the new experimental period, during which vegetable nucleic acid was ingested. The hot weather had meanwhile abated somewhat, but it is evident from the curves (figure 2) that this could not be responsible for the relatively greater urinary excretion. The subjective sensations were quite different from those experienced with animal nucleic acid. Depression was replaced by stimulation similar to that experienced by the other subjects when partaking of vegetable nucleic acid. By the fifteenth day the stimulation had become akin to a feeling of "high pressure," as though the subject's "nerves" were "highly strung." Attention became acute. There was some smarting of the conjunctiva (not noticed by the other subjects). Despite these subjective symptoms, however, blood pressure and heart rate remained normal.

In the second experiment with C.S.H. the subjective symptoms were similar to those experienced in the first experiment, but the cooler weather removed the factor of high urinary concentration to which the symptoms had previously been attributed. By the end of the animal nucleic acid period oedema of the conjunctive and puffiness under the eyes were noted, and confirmed by examination of the subject by Dr. R. F. Matters. These signs did not reappear in the vegetable nucleic acid period. The body weight at the end of the animal nucleic acid period had increased by ½ lb., which may have been due to the oedema.

Each of the subjects noted, while vegetable nucleic acid was being eaten. the presence in the urine of an abundance of crystalline material, the amount of which seemed to be out of proportion to the increase in the output of uric acid, as determined by analysis. At the end of the period these crystals appeared to be present in the urine when passed. The crystals were in the form of fine needles, giving the urine a milky appearance and an iridescent sheen when shaken. In the first experiment on C.S.H. at this stage a catheter was passed in order to ascertain whether these crystals had separated during retention of the urine in the bladder, but one hour's collection of urine obtained in this manner proved that the crystals were issuing as such from the ureters. A sample of the crystals collected from the urine of T.B.R. was dissolved in alkali and reprecipitated with excess of acetic acid several times, finally decolourized by boiling with charcoal in alkaline solution, filtering, and reprecipitating. This precipitate, when dry, proved to contain 95% of uric acid. The abundance of crystals is to be accounted for by the fact that not only the *increase* of uric acid output over basal was precipitated, but also, in consequence of the high acidity of the urine, the basal uric acid output as well. In the animal nucleic acid periods little or no precipitate was noted until the urine cooled, when phosphates were deposited.

Tests for albumin in the urine were repeatedly performed, and were uniformly negative. Nevertheless, during the nucleic acid periods Tsuchiya's reagent (an acid alcoholic solution of phospho-tungstic acid) yielded a measurable precipitate which, estimated as if it had actually been albumin, reached a maximum corresponding to about 0·5 parts per 1,000. This precipitate was not obtained with the normal urines of subjects T.B.R. and C.S.H. (H.R.M. was not tested), and undoubtedly represented a product resulting from the ingestion of nucleic acid.

During the vegetable nucleic acid period positive tests for pentose were obtained in the urine of C.S.H. (first experiment), none in the urine of T.B.R. The urine of H.R.M. and that of C.S.H. in the second experiment were not examined for pentoses.

Blood was withdrawn from subject C.S.H. 4½ hours after the midday meal on the fourth day of each four-day period during the second experiment, and the sugar content was determined before and after hydrolysis (by ½ normal hydrochloric acid for one hour at 100°C.) by the colourimetric method of Folin. The following were the results obtained:

TABLE 9. SUBJECT C.S.H., SECOND EXPERIMENT.

Blood Sugar.

Day and Period.	Estimated per cent. of Sugar in Blood. Before Hydrolysis.	After Hydrolysis for 2 hours by ½ normal acid 100°C.
Fourth day of Pre-period	0·082	0·086
Fourth day of Animal Nucleic Acid Period	0·138	0·143
Fourth day of Post-period	0·097	0·097
Fourth day of Vegetable Nucleic Acid Period	0·101	0·101
Fourth day of Post-period	0·097	0·105

Apparently a slight degree of hyperglycaemia prevailed during the period of animal nucleic acid ingestion, which may have been due to the presence in the blood of reducing substances derived from the nucleic acid, or may have been attributable to the acidosis which prevailed during the nucleic acid ingestion. As the acidosis was higher during the vegetable nucleic acid period, if the latter were the true explanation one would have expected to have observed a hyperglycaemia on the fourth day of this period also, but, as the above results show, no such effect was obtained. The idea underlying the estimation of blood sugar before and after hydrolysis by ½-normal acid was that in this way the presence of circulating nucleosides or nucleotides might be detected, but the figures afford no evidence of the presence of such substances in the blood at the times selected for the withdrawal of the specimens for analysis.

Blood counts were taken at intervals during the vegetable nucleic acid period in subject T.B.R., and during the animal nucleic acid period in subject C.S.H. (first experiment), by Dr. Florence McCoy Hill, to whom our thanks are due for this assistance. No abnormalities of erythrocyte or differential leucocyte count were found, and no leucocytosis was observed in either case.

It may be pointed out that the absence of leucocytosis following ingestion of nucleic acid throws a great deal of doubt upon the efficacy of the oral administration of "nuclein" and yeast nucleic acid, which has been recommended in some quarters for combating certain types of infection. On the other hand, the effect of yeast itself in inducing leucocytosis after oral administration has been established by Heintz and Welker (11). We must conclude, therefore, that this effect of ingesting yeast is attributable to some constituent other than nucleic acid.

BEARING OF THESE RESULTS UPON THE URIC ACID PROBLEM.

Previous authors are unanimous in their testimony that purines, whether ingested in the uncombined form, or nucleic acid, or in tissue, are only partially recoverable in the form of uric acid in the urine. Burian and Schur (6) obtained 46% recovery of urinary purines after taking 1·6 grms. of hypoxanthine. From feeding experiments they concluded that about 50% of the purines present in animal tissues were recoverable as purines from the urine after ingestion, but, as Folin, Berglund, and Derick point out (8), in some cases the recovery was much less, amounting to as little as 25% of the ingested purine. Taylor and Rose (21) recovered only half of the ingested purines from the urine. Frank and Schittenhelm (9) administered thymus nucleic acid in 10 grm. doses per day to three patients for four, four, and two days respectively. Uric acid equivalent to 5·1%, 10·5%, and 41·1% of the ingested purines was recovered from the urine. Dohrn (7) recovered only 9·7% of purines administered in the form of sweetbreads. Givens and Hunter recovered from 14% to 30% of the theoretical yield of uric acid after administration of yeast nucleic acid (10). It is noticeable in comparing the percentage recoveries reported by different authors that those experiments in which the period of administration was brief (one day or one dose), or in which the dose administered was small, obtained the highest proportion of the theoretical yield of uric acid.

The cause of this disappearance of purines has recently been made clear by the discovery of Folin, Berglund and Derick (8) that uric acid, administered intravenously, is rapidly destroyed in the blood-stream itself, only a relatively small proportion of the uric acid appearing as such, or as allantoin, in the urine. The only organ in which uric acid is stored is the kidney itself, and the effect of such storage, if the dosage of uric acid amounts to as much as 100 mg. per kilo body weight, is to cause the kidneys to "swell up to nearly twice their normal size. They become round and hard, and the surface becomes very shiny; all indicating extreme edema." Later, as the concentration of uric acid in the blood falls, due to its destruction therein, a portion of this stored-up uric acid is returned from the kidneys to the blood. Concurrently the oedema of the kidneys diminishes, but, as Folin, Berglund and Derick point out, "Transient as are the visible effects of the uric acid on the kidneys, these might well be sufficient to produce considerable and less transient effects on the function," and, as determinations of non-protein nitrogen in the blood revealed, it took a dog at least two days to recover an adequate kidney function after injection of 60 mg. of uric acid per kilogram of its body weight.

The daily ingestion of purines in our experiments was equivalent to about 4 grms. of uric acid, or 57 mgs. per kilo body weight. Admittedly ingestion by mouth and in the form of nucleic acid is not comparable with intravenous administration of uric acid itself, but if absorption of nucleic acid from the alimentary canal occurs in the form of nucleotides and nucleosides, the purines are at all events protected from destruction before absorption, so that in the course of twenty-four hours or less (since the total daily dose was administered in twelve hours) we may suppose that the whole of the administered purine entered the blood-stream in a free or combined condition. The proportion of excess phosphates in the faeces shows (Table 8) that in three out of four experiments, at all events, absorption of the administered nucleic acids was tolerably complete, while the nitrogen output shows clearly that the nitrogenous portion of the nucleic acid was generally completely absorbed. Furthermore, in place of a single injection of 60 mg. of uric acid per kilogram we had a continuous input of purines for four days in succession, equivalent to 57 mg. of uric acid per kilo per diem. It appears not improbable, therefore, that some impairment of kidney function may have resulted.

Evidence of such impairment is clearly revealed in the blood uric acid determinations which were made on subject C.S.H. during the course of the second experiment. Blood was withdrawn for analysis on the fourth day of each four-day period about four and a half hours after the midday meal. The following were the results obtained:

TABLE 10. SUBJECT C.S.H.

Blood Uric Acid.

Day and Period.	Mg. Uric Acid per 100 cc. Blood.	Urinary Uric Acid during corresponding 24 hours.
Fourth day Pre-period	2	0·32 grms.
Fourth day Animal Nucleic Acid Period ..	6	0·62 ,,
Fourth day Post-period..	5	0·43 ,,
Fourth day Vegetable Nucleic Acid Period	7	0·63 ,,
Fourth day Post-period	4·5	0·24 ,,

It will be seen that on the fourth day of the animal nucleic acid period the blood uric acid had risen to three times its level on a purine-free diet, and the urinary output was doubled. After four days of subsequent purine-free diet, the blood uric acid had fallen but slightly, although the output had fallen to only 30% above that on a purine-free diet. On the fourth day of the vegetable nucleic acid period the blood uric acid had risen to 3½ times the level of the pre-period, and the output was once more double that on a purine-free diet, but after four days of a purine-free diet, while the blood uric acid was still more than twice as concentrated as it was in the pre-period, the urinary excretion had actually fallen considerably below the level of the pre-period. This appears to indicate quite definitely that the kidneys were not functioning so efficiently at the end of the experiment as they were at the beginning. This in turn suggests an explanation of the otherwise inexplicable fact that the subjects invariably showed greater retention of nitrogen and phosphates in the *second* nucleic acid period, irrespective of the type of nucleic acid administered. It would appear that the intermediate purine-free period of four days was not sufficiently long to admit of full recovery of normal efficiency by the kidneys, so that the excretion of nitrogenous metabolites and phosphoric acid was not complete during the second nucleic acid period. The production of uric acid itself, however, was so much more rapid or complete from vegetable than from animal nucleic acid as to overcome the delay in excretion to a sufficient extent to permit the appearance of a greater output of uric acid even when vegetable nucleic acid was taken during the second period. On the one hand we appear to have a diminution of function, and on the other an excess of production, which conceivably might or might not compensate for the loss of function, but in the experiments under review appears to have done so. Under such circumstances, however, as will be clear from the following table, the disproportion between the uric acid production from the two types of nucleic acid was much less than when vegetable nucleic acid was administered during the first nucleic acid period. Presumably delay in excretion permitted a greater proportional destruction in the blood-stream. It is probably for this reason that administration of purines for short periods or in small doses leads to a greater proportional recovery of uric acid than administration in larger doses or for longer periods.

TABLE 11.

Percentage Recovery of Purines in the Form of Uric Acid from the Urine.

Subject.	Order in which the types of Nucleic Acid were administered.	Type of Nucleic Acid Administered. Vegetable.	Animal.
T.B.R.	Vegetable followed by Animal	20	5·8
H.R.M.	Vegetable followed by Animal	18	7·4
C.S.H.1.	Animal followed by Vegetable	18	9·6
C.S.H.2.	Animal followed by Vegetable	11·7	11·1

The loss of efficiency of the kidneys during the second period of nucleic acid administration is, however, shown most clearly if we compare the urinary outputs of phosphoric acid in the first and second periods *in terms of the phosphoric acid which was actually absorbed from the alimentary canal.* Presumably the excess of faecal phosphates represented phosphoric acid derived from nucleic acid which had escaped absorption. Of course, it does not follow that the other portions of the nucleic acid molecule were not absorbed, indeed, the nitrogen balances and curves of total nitrogen and urea output indicate that the nitrogen was assimilated more perfectly than the phosphoric acid. If, however, we subtract the faecal phosphates from the total phosphoric acid ingested we have an estimate of the phosphoric acid, free or combined, which actually entered the blood-stream, and we can determine what proportion of this actual intake reappeared in the urine. The following are the results:

TABLE 12.

Percentage of Absorbed Phosphoric Acid (Ingested less Faecal) which was recovered from the Urine after Nucleic Acid Administration.

Subject	Type of Nucleic Acid Administered.	First Nucleic Acid Period.	Type of Nucleic Acid Administered.	Second Nucleic Acid Period.
T.B.R.	Vegetable	85	Animal	47
H.R.M.	Vegetable	86	Animal	63
C.S.H.1.	Animal	92	Vegetable	61
C.S.H.2.	Animal	98	Vegetable	57

It appears to be quite legitimate to conclude from these figures that the excretory efficiency of the kidneys was diminished to an important extent by the administration of either type of nucleic acid in the dosages employed, and that this diminution of efficiency continued to be evident for a period exceeding four days after the discontinuance of administration.

DISCUSSION OF THE RESULTS.

Despite the diminution of the secretory efficiency of the kidneys which, as we have seen, vitiates the comparison of the excretory products of nucleic acids when administered successively to the same subject, the output of uric acid remains invariably greater when vegetable nucleic acid is eaten than when nucleic acid of animal origin takes its place in the diet. This fact admits of two explanations, that is, either the animal nucleic acid is not absorbed to nearly so great an extent as nucleic acid of vegetable origin, or, being absorbed, it is not decomposed into its constituents with the same rapidity.

The relative absorption of the two types of nucleic acid from the alimentary canal is indicated by the excess of faecal phosphates which succeeds the administration of nucleic acid. While, as we have seen (Table 8), the absorption of animal nucleic acid in three experiments out of four was inferior to the absorption of nucleic acid of vegetable origin, yet this inferiority of absorption was in no case sufficient to account for the very much greater inferiority of uric acid production.

In the four experiments taken together the average absorption of phosphoric acid from vegetable nucleic acid was 25% in excess of the absorption of phosphoric acid from animal nucleic acid, but the average production of uric acid from vegetable nucleic acid was 100% in excess of the production from animal nucleic acid. We must therefore adopt the second alternative explanation, namely, that nucleic acid of animal origin is decomposed less rapidly or less completely into its constituents than nucleic acid of vegetable origin. This conclusion recalls the finding of Levene and Medigreceanu (15), to which reference was made in the introductory part of this paper, that the various enzymes of alimentary and tissue origin decompose animal nucleic acid much more slowly than nucleic acid of vegetable origin. To this extent, therefore, the possibility indicated by their structures is confirmed, that animal and vegetable nucleic acids differ in their behaviour after ingestion.

Incidentally these results throw considerable doubt upon the theoretical foundations of the classical dietetic treatment of gout (18). If the object be to reduce the production of uric acid, animal tissues, for an equal input of nitrogen, are obviously to be preferred to vegetable tissues. Probably the beneficial effects of a vegetarian diet, if any, are attributable rather to a reduction of nitrogenous intake, and consequently of nucleic acid intake as well. Were the nitrogenous intake reduced to a like extent meat would supply a less injurious form of nucleic acid than vegetables.

So far as other metabolites are concerned, the defective efficiency of the kidneys, which is induced by the dosages of nucleic acid employed in our experiments, completely overshadows any other effect of the administration, and we cannot tell whether or to what extent the one nucleic acid may be utilized preferably to the other.

CONCLUSIONS.

1. Nucleic acids of vegetable and animal origins have been successfully administered to the same human subjects, subsisting upon an otherwise purine-free diet.

2. Animal nucleic acid is slightly less well absorbed from the alimentary canal than nucleic acid of vegetable origin, but, estimated by the faecal phosphates, the difference of absorption between the two types of nucleic acid is not great.

3. On the other hand, the output of uric acid after vegetable nucleic acid is far in excess of that excreted after eating nucleic acid of animal origin. It is inferred that nucleic acid of the vegetable type is decomposed more rapidly or more completely into its constituents than nucleic acid of the animal type.

4. The administration of the dosages of nucleic acid employed in these experiments (15 grms. per diem) leads to a demonstrable deficiency of kidney function.

5. The effect of this loss of renal activity is to induce retention of the various metabolites to such an extent as to vitiate comparisons of output when the two types of nucleic acid are successively administered. Only in the case of uric acid was the excess of production with vegetable nucleic acid sufficient to overcome the retention attributable to a previous administration of nucleic acid of the animal type.

6. Administration of nucleic acid of vegetable or animal origin by mouth leads to the appearance in the urine of some substance or substances other than

creatinine, which react with impure picric acid in alkaline solution, but do not react with picric acid which has been purified by recrystallization from benzene.

7. No leucocytosis or alteration of differential white cell count is induced by oral administration of either form of nucleic acid. The effect of yeast in inducing leucocytosis is therefore not attributable to its content of nucleic acid.

8. On an equal nitrogen intake meat is preferable to vegetables if it be desired to reduce the production of uric acid from the diet.

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BASAL METABOLISM OF MAN ON A DIET RICH IN NUCLEIC ACID

by

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(Submitted for publication 7th August, 1927.)

Some unpublished observations of T. Brailsford Robertson's on the effect of nucleic acids of plant and animal origin upon the growth of mice pointed to a possible involvement of the cellular metabolism, especially in the case of nucleic acid of animal origin, and it was assumed by Robertson that some evidence of alteration of metabolism might show itself in the urinary metabolites in a human experiment.

This latter method of investigation was adopted, and an apparent increase in creatinine excretion established by Robertson and Hicks (3). The experiment was repeated, and at the same time a careful study of the basal metabolism was carried out on one of the subjects.

Details of the main experimental work are published in another paper by Robertson, Hicks and Marston (3), and so far as the results obtained in the present work are concerned, it is only necessary to state that the experimental period of twenty days was divided into five four-day sections. During the first four days the subject was placed on a purine-free diet, which remained the basal diet throughout the whole period of the investigation. During the second four-day period 15 grms. daily of animal nucleic acid prepared from the spleen were ingested in 5 grm. doses, in addition to the non-purine dietary. The third four days were a repetition of the first purine-free period, and in the fourth period 15 grms. daily of vegetable nucleic acid were administered, the final period being a purine-free period only.

Throughout the whole investigation period the subject refrained from physical exertion of a heavy nature. All measurements were made between 7.30 and 8.30 a.m. under the recognized standard conditions, and the subject was weighed daily, without clothes, before the first meal.

The closed circuit oxygen consumption method was used, the apparatus being a spirometer with circulating fan and CO_2 absorption chamber, together with mechanism for recording respiratory movements upon a smoked drum. after the manner of Krogh (1).

In this way a graphic record of respiration of oxygen consumed, and time, in second intervals, was obtained, thus providing an accurate check which could be studied at leisure.

The purine-free dietary is illustrated by the appended diet-sheet for one day, freedom of selection from among the various ingredients being permitted.

Diet Sheet for 26/6/27.

Substance.	Grms.	Protein. Grms.	Carbohydrate. Grms.	Fat. Grms.	Calories. Total.
Bread	215·5	16·15	113	2·58	447
Butter	46·5	0·46	—	37·2	345
Cheese	63·5	15·55	1·51	20·2	265
Milk	1110·0	35·50	55·5	42·2	755
Egg	165·5	21·40	—	16·5	253
Flour	15·5	1·20	11·00	0·01	38
Sugar	30·0	—	30·0	—	110
Apple	415·0	2·40	102·0	4·0	456
Potato	277·5	4·7	49·8	—	227
				Grand Total	2,896

Note. Average daily calorific intake during experiment = 2,400 cal. Analytical data taken from Lusk (2).

Nitrogen Intake per Day.

	Purine-free Pre-period. Grms.	Animal Nucleic Acid Period. Grms.	Purine-free Pre-period. Grms.	Vegetable Nucleic Acid Period. Grms.	Purine-free Post-period. Grms.
	14·1	13·8	12·4	11·8	12·9
	15·5	13·2	14·0	13·8	14·4
	13·3	12·3	12·1	10·8	13·3
	12·4	15·4	10·6	15·2	12·3
Average	13·8	13·7	12·3	12·9	13·2

Oxygen Consumption and Basal Calorific Requirements.

Weight, 164 lbs. Height, 5 ft. 9¼ inches. Area, 1·94 sq.m. (Du Bois Formula).

Date.		Barometer (Corrected).	Temperature of Gas °C. (Average).	Time in 6 second Intervals.	Time (Average in Minutes).	Calories per sq. metre per hour.	% of Normal (37·7 cal.).	Remarks.
April	28.	743·4	28·45	32·5	3·25	38·9	+3·1	
				32·2				
				32·5				Purine free.
				32·8				
,,	29.	741·0	26·20	33·8	3·34	38·0	+0·8	
				33·2				
				33·2				
,,	30.	745·2	22·7	33·7	3·35	38·5	+2·1	15 grammes animal nucleic acid per diem.
				33·5				
May	1.	748·5	21·0	34·3	3·47	38·0	+0·8	
				35·1				
,,	2.	747·5	20·0	34·9	3·47	38·1	+1·0	
				34·5				
,,	4.	752·6	27·5	33·1	3·33	38·4	+1·8	
				33·6				
				33·2				
,,	6.	752·6	23·37	34·0	3·30	38·1	+1·0	Purine free.
				32·0				
,,	7.	745·9	24·4	33·4	3·35	38·5	+2·1	
				33·6				
,,	8.	747·0	27·5	32·9	3·30	38·4	+1·8	
				33·3				15 grammes vegetable nucleic acid per diem.
,,	11.	746·2	28·4	30·1	3·18	39·8	+5·9	
				33·0				
				32·3				
,,	12.	753	23·4	36·4	3·68	35·6	—5·6	Purine free.
				37·2				
,,	14.	748	25·0	36·9	3·69	35·0	—4·5	
				38·8				
				35·0				

Note. On May 10, 11, and 12 an attack of influenza supervened. The highest B.M.R. corresponds to the height of the attack; the lower figures following may be due to post-influenzal debility.

The results indicate that within 1 calorie of the average normal for the experimental subject the method would not disclose any alteration in heat production. After the infection the figures are less reliable, and have been excluded in the determination of the average basal figure, but even these latter results do not fall outside the normal fluctuation in the basal rate.

It is more than probable that the experimental period was too short to detect any alteration in metabolism. which is suggested by the life period results obtained by Robertson with mice, and which will be shortly reported in this journal. The changes in creatinine production, previously referred to, have been shown to be due to some substance which gives Jaffe's test with impure picric acid, and which was increased in amount by the ingestion of nucleic acid (3), so that the present results are in harmony with that conclusion, the creatinine excretion in the present experiment being unaltered when benzene crystallized picric acid was used as the reagent.

My thanks are due to my wife for assistance in securing the basal metabolic measurements.

SUMMARY.

1. Basal metabolism of a human subject on a purine-free diet, with the addition of nucleic acid of plant and animal origin, has been studied.

2. So far as these determinations are concerned, no significant alteration of basal metabolism results from ingestion of nucleic acid, whether of animal or vegetable origin.

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COMPENSATORY RENAL HYPERTROPHY

by

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(Submitted for publication 18th July, 1927.)

It is a long-known and well-established fact that after unilateral nephrectomy the surviving kidney undergoes enlargement. A similar enlargement of one kidney also takes places when unilateral hydronephrosis is produced in its fellow by ligation of the ureter. Arataki (1926), who gives an excellent bibliography of the more recent of the numerous papers describing these changes, concludes from his own studies on the white rat, and in agreement with the majority of earlier workers on other species, that the enlargement is a true hypertrophy of the secreting units of the kidney, accompanied by hyperplasia of the supporting tissue, and to some extent of the cells lining the tubules.

The cause of the hypertrophy is still under discussion, and more than one factor is doubtless involved. The surviving kidney has at all events in the early period after the operation an increased blood supply. Sacerdotti (1896) observed no enlargement of the surviving kidney in fasting animals which had been subjected to unilateral nephrectomy, but found that the blood from animals in which both kidneys had been removed caused enlargement of the kidneys when repeatedly injected into normal animals after removing an equivalent amount of blood so as to avoid altering the blood volume. Smith and Moise (1927) have been able to demonstrate increase in the rate of enlargement of the surviving kidney in rats fed on high protein diets, and such diets alone tend in intact rats to cause renal enlargement (Osborne, Mendel, Park, and Winternitz, 1925). It is clear, then, that increased work which has to be done by the surviving kidney is the main factor in causing hypertrophy. It seemed possible, however, that in diseased conditions an added stimulus to hypertrophy might be furnished by a specific stimulant product of the autolysis of damaged renal tissue.

In some experiments on compensatory hypertrophy of the suprarenals in the rabbit, Boycott and Kellaway (1924) attempted to test this general conception by comparison of the effects of excision with those of tying off one adrenal. The results, however, were wholly negative. The comparison of the degree of hypertrophy in animals in which one kidney has been excised with others in which a hydronephrosis has been produced by ligation of the ureter will not suffice to settle this point, since the stimulus to hypertrophy from piling up of products of metabolism in the blood stream is not necessarily identical in both cases. We have therefore compared the extent of hypertrophy in three series of animals. In one group unilateral nephrectomy was performed in some animals, ligature of one ureter in others, while the remainder were unoperated controls. In the second the results of unilateral nephrectomy were compared with the hypertrophy produced when in addition repeated subcutaneous injections of fresh-minced homologous renal tissue were made. In the final group the extent of hypertrophy following nephrectomy was compared with that which follows the ligation of the pedicle of one kidney, a process which results in complete atrophy on the operated side within 70 days, and does not appear to affect the general health of the animal.

METHODS.

The animals used were guinea-pigs, 158 in number. Group 1 (a) comprised 21 guinea-pigs in which the left kidney had been excised, 13 in which hydronephrosis was produced by ligation of the left ureter, and 14 control animals, in which no operation was performed. These were studied after 60 days. In Group 1 (b), of 27 animals, in 14 the left kidney was removed, and in 13 the left ureter was ligated. The animals were killed 90 days after operation. In all the animals of Group 2 (42 in number) the left kidney was removed, and in 21 of these weekly injections of fresh guinea-pig kidney tissue were given subcutaneously. These were killed 75 to 78 days after operation, except 3 killed on the 95th day. In Group 3 (of 41 guinea-pigs) in 20 the left kidney was removed, and in 21 the vessels and ureter were tied off *en masse* at the hilum. In this group the animals were nearly all killed after 76 days. All these experiments were so arranged that animals in which the results were to be compared came from the same litters.

The operative procedures were carried out by one of us (C.H.K.) under full ether anaesthesia and with aseptic precautions. The animals were from 14 to 30 days old when operated upon, and weighed from 110 to 280 grms. The younger animals were returned to their mothers after operation, and nourished by suckling until a month old. A short incision was made in the left flank, the peritoneum was opened, and the kidney delivered. When the ureter was ligated this was done immediately distal to the pelvis of the kidney. For nephrectomy or simple tying off of the kidney alone the vessels and ureter were ligated *en masse* at the hilum after the capsule had been stripped back, and in the female, before delivering the kidney for decapsulation, it was necessary to divide between ligatures the suspensory ligament of the ovary to avoid post-operative haemorrhage from the artery accompanying it. The time occupied in the operations varied from 7 to 10 minutes, and the immediate mortality was 6·4%. All wounds healed by first intention. When injections of fresh renal tissue were made, a guinea-pig was killed, the kidneys removed aseptically, minced in a tumour mincer, and injected in doses of 0·05 c.cm. with a Bashford syringe. This procedure was carried out with strict aseptic precautions. The animals were kept, one only in each cage, under uniform conditions, and the diet, which was ample, consisted of bran, cabbage, lettuce leaves, and occasionally carrots.

The temperature of the animal houses was regulated when necessary by steam radiators, and the animals were regularly exposed to sunlight for similar periods.

The guinea-pigs were autopsied in the same order as that in which they had been operated on, controls being killed on the same day as their litter mates. The technique was as follows. After fasting over night for 12–18 hours the animal was weighed, killed by a blow on the head, and then bled out as far as possible by dividing the vessels in the neck. The thoracic and abdominal cavities were opened and the viscera removed, stored in a moist air chamber, and weighed at leisure. The alimentary canal, gall bladder, and urinary bladder were weighed after their contents had been washed out. The final body weight used in calculations was obtained by adding the weight of the organs to that of the eviscerated carcase.

The dry weight of the organs was also obtained by drying them in an oven at 70°C. for 72 hours, and finally in a desiccator to constant weight.

RESULTS.

The results of the experiments are summarized in Tables 1 (a), 1 (b), 2, and 3. In these the weight of the body at death, and the wet and dry weights of the surviving kidney, heart, and liver, are given for each animal, together with the ratios (× 1,000) of the weights of the organs in grammes to the body weight in grammes. The mean values of the ratios in each group and of the body weights have been calculated, and in the former case the standard deviation and standard error of the mean have been determined wherever the number of observations has not been too small for this purpose.

The operations did not cause any definite symptoms, save in guinea-pig 1, Table 1 (a), which on the 60th day after nephrectomy had a series of fits and a blood urea of 87 mgm. per 100 c.cm. Miss Beryl Splatt determined the blood urea values in 30 animals of Groups 1 (a) and (b) at death. Sixty days after nephrectomy the mean value was 53 mgm. per 100 c.cm. (8 observations), 90 days after nephrectomy, 43 mgm. (8 observations), 60 days after unilateral ligation of the ureter, 55 mgm. (5 observations), and 90 days after ligation of the ureter 47 mgm. (9 observations). The mean value for the blood urea in a series of considerably older normal guinea-pigs was 56 mgm. per 100 c.cm. These results suggest some improvement in function with lengthening of the period of survival, but are too few to be significant, except in so far as they indicate that there was no gross impairment of function in the animals so tested apart from the case mentioned above.

In Group 1 (a) the mean value of the ratio (× 1,000) of the wet weight of the surviving kidney to the body weight was 7·04 ± ·11 in the nephrectomy sub-group, 6·51 in the hydro-nephrotic sub-group, and 4·69 ± ·10 in the corresponding controls. The mean ratios (× 1,000) of the dry weight to the body weight were 1·44 ± ·03, 1·35 and 0·94 ± ·02, and indicate that there has been no appreciable increase of the water content in the surviving kidney in the hydronephrotic or nephrectomized sub-group. Compensatory hypertrophy of the surviving kidney is definitely greater in the nephrectomized than in the hydronephrotic guinea-pigs at this period after operation. The mean body weights in the three sub-groups, 362, 369, and 365 gms., were so close that a less degree of hypertrophy in the hydronephrotic group cannot be attributed to nutritional causes. In the absence of evidence that the stimulus of increased load on the surviving kidney is greater in the case of animals in which one kidney has been removed, though the kidney may have a somewhat greater vascular supply during the early period after the operation, the observed facts might even be held to suggest a hypothesis diametrically opposite to that which we were testing, namely, that products of renal tissue autolysis actively inhibit hypertrophy of the surviving kidney.

In this group the mean of the values of the ratio (× 1,000) of the wet weight of the heart to the body weight was 3·50 ± ·04 in the nephrectomized, 3·36 ± ·06 in the hydronephrotic, and 3·33 ± ·07 in the control group. The corresponding dry weight ratios were ·59 ± ·02, ·59 and ·66 ± ·02, and indicate a somewhat greater water content in the heart muscle in the two operated sub-groups. These figures clearly show that in young guinea-pigs the compensatory hypertrophy of the surviving kidney, which occurs following removal of one kidney or ligation of one ureter, is unaccompanied by hypertrophy of the heart. Grawitz and Israel (1897). among the earlier workers, noted that hypertrophy of the heart only follows extirpation of one kidney in the case of the older animals.

The mean of the rations (× 1,000) of the wet weight of the liver to body weight was 31·33 ± ·46 in the nephrectomized sub-group, 29·69 ± 1·46 in the hydronephrotic, and 30·00 ± ·77 in the control sub-group. The corresponding means of the dry weight ratios were 7·30 ± ·33, 7·30 and 7·00 ± ·25.

The results of Group 1 (b), examined 90 days after operation, confirm the findings in Group 1 (a). The mean ratios (× 1,000) of wet weight of the surviving kidney to body weight were 6·00 ± ·14 in the nephrectomized, and 5·71 ± ·08 in animals after ligation of one ureter, the corresponding dry weight ratios being 1·15 ± ·01 and 1·10 ± ·02. The difference between these values, unlike the observed difference at the 60th day, is only just outside the standard error, and renders it probable that the larger difference at the earlier period is due to greater vascularity in and possibly increased functional demand upon the surviving kidney of the nephrectomized animals in the early days after operation. The means of the wet ratios (× 1,000) of the heart to body weight were 3·07 ± ·06 and 2·96 ± ·03, and those of the liver were 29·14 ± ·95 and 26·62 ± ·84.

In Group 2 an attempt has been made to evaluate the effect of the subcutaneous injection of fresh sterile renal tissue on the compensatory hypertrophy of the surviving kidney. In the control sub-group, from which the left kidney had been removed, but no injections made, the means of the wet and dry weight ratios (× 1,000) were 7·21 ± ·16 and 1·56 ± ·03 for the surviving kidney, as compared with the sub-group in which removal of the left kidney was followed by a series of injections of renal tissue, and in which the values were 7·09 ± ·16 and 1·52 ± ·03. The difference observed is within the standard error of the mean, so that no effect has been demonstrated. That no impairment of the general health or nutrition of the animals was caused by the injections is indicated by the mean body weights, which were 347 in the uninjected and 351 in the injected sub-group of guinea-pigs. It might be argued that the dosage of renal tissue injected (·05 c.cm weekly) was too small to produce any result. A final experiment was therefore made in Group 3, in which the same comparisons were instituted between a sub-group in which nephrectomy had been performed and another in which the pedicle of the left kidney had been tied off *en masse* and the organ left *in situ*. These last animals, examined after 76 days, had almost the same mean body weight (439 grms.) as that of the guinea-pigs in which the kidney had been removed after ligation of the pedicle (436 grms.). At this time the tied-off kidney had atrophied, so that about 1 grm. (the average weight of the left kidney when removed at operation) of renal tissue had been autolyzed and absorbed during this period.

In the control group of nephrectomized animals the means of the ratios (× 1,000) of wet and dry weights to body weight were 7·20 ± ·10 and 1·70 ± ·05 for the surviving kidney as compared with the corresponding values in the groups in which the kidney was tied off, 6·96 ± ·07 and 1·62 ± ·03. No significant difference is detectable here.

Whatever influence products of autolysis of differentiated tissue may have in causing new growth of fibrous tissue, it is clear that they neither increase nor decrease the hypertrophy of the surviving kidney. The figures in Table 3 likewise exclude the possibility of their causing hypertrophy of the heart or liver.

CONCLUSIONS.

1. Specific products of autolysis of renal tissue have no influence in causing compensatory overgrowth of the surviving kidney in the guinea-pig following unilateral nephrectomy or ligation of a single ureter.

2. No cardiac or hepatic overgrowth is observed in young guinea-pigs following these procedures.

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SERIES 1.
Group 1 (a).
Sixty Days.

Excision of Left Kidney.

Number of Animal.	Number of Litter.	Sex.	Time after Operation.	Body Weight at Death.	Weight of Organs in Grammes with their Ratios (× 1,000) to Body Weight in Grammes.											
					Kidney.				Heart.				Liver.			
					Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).	
					Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.
1	I	M	60	498	2·87	—	5·76	—	1·68	—	3·37	—	14·83	—	29·78	—
3	II	M	60	436	3·01	—	6·96	—	1·77	—	4·06	—	13·26	—	30·41	—
4	II	F	60	353	2·40	—	6·81	—	1·20	—	3·40	—	10·22	—	28·95	—
6	III	M	60	339	2·20	—	6·50	—	1·16	—	3·42	—	9·68	—	28·56	—
8	IV	M	60	432	2·72	—	6·30	—	1·47	—	3·41	—	13·22	—	30·60	—
9	IV	M	60	415	2·77	—	6·68	—	1·31	—	3·16	—	13·12	—	31·61	—
39	XVIII	F	65	416	2·45	0·509	5·89	1·22	1·31	0·212	3·15	0·51	13·56	4·220	32·59	10·14
43	XIX	F	65	395	2·58	0·541	6·53	1·37	1·36	0·234	3·44	0·59	13·77	4·400	34·86	11·14
46	XIX	M	65	332	2·29	0·388	6·89	1·17	1·23	0·155	3·71	0·47	11·18	2·986	33·67	8·99
51	XXI	F	60	276	2·10	0·413	7·61	1·50	0·90	0·151	3·26	0·55	7·96	1·599	28·84	5·79
52	XXII	F	60	296	2·54	0·550	8·58	1·86	0·98	0·106	3·31	0·36	7·97	1·861	26·93	6·29
56	XXIII	M	60	402	2·85	0·551	7·09	1·37	1·35	0·235	3·36	0·59	11·88	2·232	29·55	5·55
57	XXIV	F	60	340	2·58	0·547	7·59	1·61	1·15	0·221	3·38	0·65	10·76	2·138	31·65	6·29
63	XXVI	M	60	320	2·30	0·468	7·19	1·46	1 10	0·210	3·44	0·66	9·54	1·636	29·81	5·11
66	XXVIII	F	60	360	2·38	0·466	6·61	1·29	1·23	0·236	3 42	0·66	11·88	2·138	33·00	8·94
69	XXIX	M	60	314	2 48	0·502	7·89	1·57	1·02	0·212	3·25	0·68	11·88	2·561	37·83	8·16
72	XXX	F	60	325	2·18	0·416	6 71	1·28	1·23	0·238	3·78	0·37	8·78	1·520	27·02	4·68
73	XXX	M	60	366	2·50	0·513	6·83	1·40	1·32	0·238	3·61	0·65	12·88	2·768	35·19	7·56
75	XXXI	F	60	337	2·37	0·479	7·03	1·42	1·32	0·268	3·92	0·80	11·55	2·613	34·27	7·75
76	XXXI	M	60	324	2·63	0·525	8·12	1·62	1·20	0·255	3·71	0·79	9·74	1·876	30·06	5·790
79	XXXII	F	60	328	2·68	0·522	8·17	1·59	1·21	0·252	3·69	0·77	12·17	2·468	37·10	7·52
Mean				362			7·04	1·44			3·50	0·59			31·33	7·30
Standard Deviation							0·729	0·182			0·247	0·122			3·106	1·91
Probable Error of Mean							±0·11	±0·03			±0·04	±0·02			±0·46	±0·33

Group 1 (a) (continued).
Sixty Days.

Ligation of Left Ureter.

Number of Animal.	Number of Litter.	Sex.	Time after Operation.	Body Weight at Death.	Weight of Organs in Grammes with their Ratios (× 1,000) to Body Weight in Grammes.											
					Kidney.				Heart.				Liver.			
					Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).	
					Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.
2	I	M	60	425	2·79	—	—	—	1·54	—	3·63	—	12·11	—	28·49	—
5	II	M	60	410	2·65	—	—	—	1·55	—	3·78	—	13·01	—	31·73	—
7	III	M	60	357	2·39	—	—	—	1·20	—	3·36	—	9·59	—	26·86	—
10	IV	M	60	382	2·51	—	—	—	1·22	—	3·19	—	11·37	—	29·76	—
40	XVIII	F	65	330	2·18	0·433	6·61	1·31	1·06	0·169	3·22	0·51	9·46	2·801	28·67	8·49
44	XIX	F	65	306	1·90	0·392	6·21	1·28	1·06	0·118	3·47	0·39	7·52	1·488	24·57	4·86
48	XX	M	60	421	2·37	0·502	5·63	1·19	1·32	0·152	3·13	0·36	10·65	2·784	25·29	6·61
50	XXI	F	60	345	2·39	0·512	6·93	1·48	1·32	0·261	3·83	0·76	9·75	2·012	28·26	5·83
53	XXII	F	60	386	2·48	0·538	6·43	1·39	1·20	0·196	3·11	0·51	10·17	2·318	26·35	6·01
54	XXIII	F	60	350	2·25	0·496	6·43	1·42	1·07	0·188	3·06	0·54	9·57	2·088	27·34	5·68
58	XXIV	F	60	343	2·49	0·528	7·26	1·54	1·22	0·241	3·56	0·70	11·56	2·324	33·70	6·78
59	XXV	F	60	384	2·45	0·488	6·38	1·27	1·45	0·276	3·78	0·79	14·17	3·006	36·90	7·83
61	XXVI	F	60	355	2·21	0·478	6·23	1·35	0·99	—	2·79	—	12·72	2·586	35·83	7·28
Mean				369			6·51	1·35			3·36	0·59			29·69	7·30
Standard Deviation											0·329				3·667	
Probable Error of Mean											±0·06				±1·464	
Controls: Right Kidney.																
41	XVIII	M	65	428	1·87	0·386	4·37	0·90	1·25	0·220	2·92	0·51	14·30	4·495	33·41	10·50
42	XIX	F	65	400	1·44	0·293	3·60	0·73	1·05	0·284	2·63	0·71	9·59	2·930	23·98	7·33
45	XIX	M	65	275	1·27	0·261	4·62	0·95	0·99	0·172	3·59	0·63	7·87	2·312	28·62	8·41
47	XX	M	60	438	1·71	0·351	3·90	0·80	1·30	0·188	2·97	0·43	11·12	2·986	25·39	6·59
49	XXI	F	60	383	1·69	0·382	4·38	1·00	1·20	0·302	3·14	0·79	11·22	2·518	29·29	6·78
55	XXIII	M	60	356	1·70	0·372	4·77	1·05	1·08	0·210	3·03	0·59	8·90	1·887	25·00	5·30
60	XXV	M	60	444	2·00	0·389	4·50	0·88	1·62	0·336	3·65	0·76	16·00	3·868	36·04	8·71
62	XXVI	M	60	332	1·80	0·360	5·42	1·08	1·06	0·202	3·19	0·61	10·12	2·005	30·48	6·04

Group 1 (a) (continued).

Controls: Right Kidney (continued).

Sixty Days.

Weight of Organs in Grammes with their Ratios (× 1,000) to Body Weight in Grammes.

Number of Animal.	Number of Litter.	Sex.	Time after Operation.	Body Weight at Death.	Kidney. Weight. Wet.	Kidney. Weight. Dry.	Kidney. Ratio (× 1,000). Wet.	Kidney. Ratio (× 1,000). Dry.	Heart. Weight. Wet.	Heart. Weight. Dry.	Heart. Ratio (× 1,000). Wet.	Heart. Ratio (× 1,000). Dry.	Liver. Weight. Wet.	Liver. Weight. Dry.	Liver. Ratio (× 1,000). Wet.	Liver. Ratio (× 1,000). Dry.
65	**XXVII**	**M**	60	375	1·85	0·352	4·93	0·94	1·15	0·246	3·07	0·66	11·58	2·112	30·88	5·63
68	**XXIX**	**M**	60	349	1·90	0·328	5·44	0·94	1·16	0·218	3·33	0·63	12·86	2·488	36·85	7·13
70	**XXX**	**M**	60	342	1·58	0·312	4·62	0·91	1·30	0·261	3·80	0·76	8·79	1·762	25·70	5·15
74	**XXX**	**F**	60	339	1·50	0·299	4·42	0·88	1·22	0·224	3·60	0·66	10·76	2·132	31·74	6·29
78	**XXXI**	**F**	60	327	1·78	0·326	5·44	0·10	1·29	0·251	3·95	0·77	10·36	2·068	31·68	6·32
80	**XXXII**	**F**	60	319	1·68	0·326	5·26	1·02	1·20	0·236	3·77	0·74	9·52	1·718	29·84	5·39
Mean				365			4·69	0·94			3·33	0·66			30·00	7·00
Standard Deviation							0·549	0·084			0·382	0·093			4·278	1·40
Probable Error of Mean							±0·10	±0·02			±0·07	±0·02			±0·77	±0·25

Group 1 (b).

Excision of Left Kidney.

Ninety Days.

Number of Animal.	Number of Litter.	Sex.	Time after Operation.	Body Weight at Death.	Kidney. Weight. Wet.	Kidney. Weight. Dry.	Kidney. Ratio (× 1,000). Wet.	Kidney. Ratio (× 1,000). Dry.	Heart. Weight. Wet.	Heart. Weight. Dry.	Heart. Ratio (× 1,000). Wet.	Heart. Ratio (× 1,000). Dry.	Liver. Weight. Wet.	Liver. Weight. Dry.	Liver. Ratio (× 1,000). Wet.	Liver. Ratio (× 1,000). Dry.
11	V	F	93	432	2·41	0·465	5·58	1·08	1·25	0·198	2·89	0·46	13·02	3·982	30·14	9·22
12	V	M	93	471	2·50	0·515	5·31	1 09	1·49	0·268	3·17	0·57	13·85	3·774	29·41	8·01
15	VI	M	93	496	2·75	0·515	5 55	1·04	1·68	0·230	3·38	0·46	14·21	4·340	28·65	8·75
17	VII	M	93	461	2·41	0·472	5·23	1·02	1·14	0·197	2·47	0·43	11·39	3·478	24·71	7·54
18	VIII	M	92	387	2·91	0·542	7·52	1·40	1·48	0·237	3·83	0·61	14·02	4·250	36·23	10·78
22	IX	M	92	340	2·70	0·442	7·94	1 30	1·22	—	3·59	—	13·20	3·694	38·82	10·87
23	XIII	M	82	470	3·17	0·505	6 74	1·08	1·42	0·260	3·06	0 52	10·59	3·051	23·75	6·88
24	X	M	92	464	2·44	0·467	5·32	1·01	1·42	0 240	2·88	0·47	11·02	3·194	27·65	7·07
27	XI	F	92	340	2·23	0·426	6·56	1·25	0·98	0·161	3·05	0·50	9·40	2·402	21·81	6·15
28	XII	F	92	442	2·61	0·522	5 91	1·18	1·35	0·223	3·02	0·55	9·64	2·718	22·53	6·49
31	XIV	F	92	468	2·68	0·556	5·73	1·19	1·45	0·290	3·09	0·63	11·85	3·725	25·32	7·96
33	XV	F	92	481	2·69	0·582	5 59	1 21	1·33	0·271	2·77	0·56	17·16	5·022	35·67	10·44
35	XVI	F	91	430	2·69	0·515	6·26	1·20	1·36	0·172	3·16	0·40	12·38	2·488	28·79	5·79
37	XVII	F	91	470	2·58	0·512	5·49	1·09	1·31	0·246	2·79	0·52	15·38	3·026	32·72	6·44
Mean				439			6·00	1·15			3·07	0·53			29·14	8·00
Standard Deviation							0·782	0·125			0·336	0·074			5·273	1·64
Probable Error of Mean							±0·14	±0·01			±0·06	±0·01			±0·95	±0·29

Ligation of Left Ureter.

Group 1 (b) (continued).

Ninety Days.

Number of Animal.	Number of Litter.	Sex.	Time after Operation.	Body Weight at Death.	Weight of Organs in Grammes with their Ratios (× 1,000) to Body Weight in Grammes.											
					Kidney.				Heart.				Liver.			
					Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).	
					Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.
13	VI	F	93	402	2·15	0·447	5·35	1·11	1·20	0·148	2·98	0·37	11·98	3·552	29·80	8·84
14	VI	M	93	418	2·30	0·432	5·50	1·03	1·20	0·184	2·88	0·44	14·72	3·908	35·22	9·35
16	VII	M	93	500	2·80	0·474	5·60	0·95	1·53	0·148	3·06	0·30	16·51	4·486	33·02	8·97
19	VIII	F	93	406	2·22	0·470	5·47	1·16	1·13	0·192	2·78	0·47	8·87	2·598	21·85	6·40
20	VIII	F	92	388	2·10	0·393	5·41	1·01	1·15	0·206	2·96	0·53	11·65	3·240	30·03	8·35
21	IX	F	93	420	2·47	0·462	5·88	1·10	1·24	0·218	2·95	0·52	9·71	2·925	23·12	6·96
25	X	M	92	505	2·97	0·551	5·88	1·09	1·59	0·272	3·15	0·54	12·10	3·152	23·96	6·24
26	XI	M	92	460	2·58	0·498	5·61	1·08	1·38	0·220	3·00	0·48	9·93	3·073	21·59	6·69
29	XII	M	92	490	2·60	0·535	5·31	1·09	1·38	0·247	2·82	0·48	10·45	3·775	21·33	7·70
32	XIV	M	92	375	2·67	0·510	7·12	1·36	1·20	0·223	3·20	0·60	10·31	2·930	27·49	7·81
34	XV	F	92	394	2·50	0·498	6·34	1·26	1·18	0·208	2·99	0·53	9·98	2·010	25·33	5·10
36	XVI	F	91	490	2·66	0·538	5·43	1·10	1·39	0·237	2·84	0·48	14·77	3·126	30·14	6·38
38	XVII	M	91	530	2·78	0·558	5·25	1·05	1·52	0·218	2·87	0·41	16·88	3·487	31·85	6·58
Mean				444			5·71	1·10			2·96	0·47			26·62	7·27
Standard Deviation							0·451	0·089			0·139	0·092			4·483	1·12
Probable Error of Mean							±0·08	±0·02			±0·03	±0·02			±0·84	±0·21

Excision of Left Kidney.

SERIES II.
Group 2.

Weight of Organs in Grammes with their Ratios (× 1,000) to Body Weight in Grammes.

Number of Animal.	Number of Litter.	Sex.	Time after Operation.	Body Weight at Death.	Kidney.				Heart.				Liver.			
					Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).	
					Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.
82	XXXIII	F	—	412	2·35	0·480	5·70	1·16	1·36	0·244	3·30	0·59	12·76	2·471	30·97	6·00
83	XXXIV	F	—	285	2·02	0·482	7·07	1·68	1·05	0·202	3·69	0·71	9·19	2·063	32·25	7·24
85	XXXV	M	—	363	2·90	0·622	7·99	1·71	1·34	0·235	3·69	0·65	15·22	3·167	41·93	8·72
88	XXXVI	F	—	387	2·79	0·586	6·98	1·53	1·32	0·266	3·41	0·69	13·65	2·448	35·27	6·33
89	XXXVII	F	—	395	2·26	0·535	5·72	1·36	1·40	0·288	3·55	0·73	14·25	3·164	36·08	8·01
94	XXXIX	F	—	320	2·18	0·474	6·81	1·48	1·21	0·254	3·78	0·79	10·50	2·228	32·81	6·96
96	XL	F	—	320	2·30	0·488	7·19	1·52	1·21	0·237	3·78	0·74	12·00	2·586	37·50	8·08
99	XLI	M	—	410	2·77	0·602	6·76	1·47	1·40	0·346	3·42	0·85	12·07	3·122	29·44	7·62
100	XLII	M	—	346	2·67	0·559	7·72	1·62	1·38	0·326	3·99	0·95	13·95	3·264	40·32	9·43
103	XLIII	M	—	365	2·84	0·576	7·78	1·58	1·29	0·312	3·53	0·86	10·07	2·516	27·59	6·89
107	XLIV	F	—	200	2·25	0·440	11·25	2·20	0·90	0·250	4·50	1·25	7·20	2·461	36·00	12·31
108	XLIV	F	—	315	2·25	0·468	7·14	1·48	1·23	0·312	3·91	0·99	9·30	2·336	29·52	7·42
109	XLV	F	—	360	2·38	0·520	6·61	1·45	1·06	0·210	2·95	0·58	10·15	2·388	28·19	6·63
112	XLVI	F	—	322	2·45	0·572	7·61	1·78	1·10	0·232	3·42	0·72	11·55	2·916	35·87	9·06
113	XLVI	M	—	344	2·40	0·540	6·98	1·57	1·12	0·223	3·25	0·65	9·52	2·161	27·67	6·28
116	XLVII	M	—	345	2·40	0·496	6·96	1·44	1·25	0·261	3·62	0·76	12·40	2·812	35·94	8·15
119	XLVIII	F	—	340	2·45	0·598	7·21	1·76	1·26	0·218	3·71	0·64	9·52	1·961	28·00	5·77
121	XLIX	M	—	390	2·39	—	6·13	1·25	1·25	0·258	3·21	0·66	12·48	2·563	32·00	6·57
122	L	F	—	400	2·81	—	7·03	1·44	1·35	0·279	3·38	0·70	15·93	3·324	39·83	8·31
126	LI	M	—	340	2·66	—	7·02	1·59	1·48	0·375	4·36	1·11	13·35	2·688	39·27	7·91
127	LI	F	—	330	2·46	—	7·46	1·87	1·38	0·279	4·18	0·85	14·48	2·988	43·88	9·06
Mean				347			7·21	1·56			3·64	0·78			34·38	7·79
Standard Deviation							1·091	0·222			0·356	0·173			4·764	1·61
Probable Error of Mean							±0·16	±0·03			±0·05	±0·03			±0·70	±0·24

SERIES II.

Group 2 (continued).

Excision of Left Kidney with Injection of Renal Substance.

Weight of Organs in Grammes with their Ratios (× 1,000) to Body Weight in Grammes.

Number of Animal.	Number of Litter.	Sex.	Time after Operation.	Body Weight at Death.	Kidney.				Heart.				Liver.			
					Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).	
					Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.
81	XXXIII	F	95	350	2·35	0·498	6·71	1·42	1·22	0·216	3·49	0·62	11·78	2·067	33·66	5·91
84	XXXIV	M	95	463	2·85	0·633	6·16	1·37	1·59	0·269	3·44	0·58	14·88	2·885	32·14	6·23
87	XXXVI	F	95	422	2·76	0·598	6·54	1·41	1·34	0·225	3·17	0·53	12·78	2·286	30·28	5·42
90	XXXVII	F	78	387	2·32	0·493	5·99	1·28	1·19	0·243	3·08	0·63	14·36	2·874	37·11	7·43
92	XXXVIII	M	78	403	2·34	0·516	5·81	1·28	1·40	0·237	3·48	0·59	12·22	2·136	30·32	5·30
93	XXXIX	F	77	310	2·28	0·482	7·36	1·56	1·15	0·225	3·71	0·73	10·56	1·989	34·07	6·42
97	XL	F	77	350	2·34	0·486	6·69	1·39	1·17	0·247	3·35	0·71	10·02	2·165	28·61	6·19
98	XLI	F	75	392	2·61	0·556	6·66	1·42	1·29	0·336	3·29	0·86	9·96	2·483	25·41	6·33
101	XLII	M	75	360	2·72	0·548	7·56	1·56	1·14	0·361	3·17	1·00	10·88	2·862	30·22	7·95
102	XLII	M	75	224	2·41	0·494	10·76	2·20	0·97	0·244	4·33	1·09	6·90	1·682	30·80	7·51
104	XLIII	M	75	320	2·48	0·512	7·75	1·60	1·24	0·312	3·87	0·98	9·25	2·386	28·91	7·46
106	XLIV	F	75	345	2·22	0·503	6·44	1·46	1·20	0·298	3·48	0·86	10·29	2·566	29·83	7·44
110	XLV	M	76	440	2·90	0·596	6·59	1·355	1·35	0·278	3·07	0·63	14·73	2·988	33·48	6·79
111	XLV	F	76	314	2·00	0·422	6·37	1·344	1 10	0·243	3·51	0·78	9·72	1·916	30·95	6·10
114	XLVI	M	75	370	2·54	0·532	6·87	1·439	1·18	0·231	3·19	0·63	9·10	2·016	24·59	5·45
115	XLVII	M	76	294	2·45	0·512	8·33	1·742	1·10	0·236	3·75	0·80	9·06	1·910	30·82	6·50
117	XLVIII	F	76	342	2·37	0·596	6·93	1·742	1·38	0·352	4·03	1·03	12·10	3·112	35·38	9·10
120	XLIX	F	76	322	2·02	0·420	6·27	1·303	1·08	0·221	3·36	0·69	9·94	1·983	30·87	6·10
123	L	F	76	284	2·38	0·488	8·38	1·718	1·15	0·238	4·05	0·84	10·25	2·110	36·09	7·43
124	L	F	76	300	2·63	0·536	8·77	1·787	1·32	0·264	4·40	0·88	11·38	2·278	37·93	7·60
125	LI	M	76	370	2·58	0·520	6·98	1·406	1·55	0·332	4·19	0·90	15·56	3·168	42·05	8·56
Mean				351			7·09	1·52			3·59	0·78			32·29	6·88
Standard Deviation							1·087	0·222			0·421	0·152			4·012	0·995
Probable Error of Mean							±0·16	±0·03			±0·06	±0·02			±0·59	±0·15

SERIES III.

Group 3.

Excision of Left Kidney.

Weight of Organs in Grammes with their Ratios (× 1,000) to Body Weight in Grammes.

Number of Animal.	Number of Litter.	Sex.	Time after Operation.	Body Weight at Death.	Kidney.				Heart.				Liver.			
					Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).	
					Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.
1	LII	M	76	452	2·95	0·608	6·53	1·34	1·76	0·382	3·90	0·845	20·65	4·388	45·69	9·71
4	LIII	M	76	495	3·25	0·662	6·56	1·34	1·86	0·388	3·76	0·785	18·40	3·728	37·17	7·53
6	LIV	M	76	416	3·02	0·612	7·26	1·47	1·82	0·387	4·38	0·931	14·85	2·989	35·69	7·19
8	LV	M	76	427	2·89	0·591	6·77	1·39	1·68	0·366	3·94	0·858	14·72	2·966	34·47	6·95
10	LVI	M	76	448	2·92	0·591	6·52	1·32	1·70	0·356	3·80	0·795	14·35	2·986	32·03	6·67
12	LVII	F	76	468	2·80	0·566	5·98	1·21	1·82	0·378	3·89	0·807	15·16	3·121	32·39	6·67
14	LVIII	F	76	418	3·00	0·382	7·18	1·46	1·78	0·382	4·26	0·914	16·38	3·318	39·18	7·94
16	LIX	M	76	495	3·62	0·377	7·31	1·46	1·88	0·377	3·80	0·763	24·70	4·988	49·89	10·08
20	LX	F	76	428	3·08	0·786	7·18	1·84	1·74	0·489	4·07	1·142	16·12	4·212	37·67	9·48
21	LXI	F	76	398	2·97	0·755	7·46	1·89	1·86	0·488	4·70	1·226	11·66	2·996	29·29	7·53
23	LXII	F	76	446	3·18	0·796	7·13	1·79	1·89	0·499	4·28	1·118	16·32	4·122	36·59	9·24
26	LXIII	F	76	378	2·60	0·671	6·88	1·78	1·66	0·418	4·39	1·106	15·52	3·902	41·06	10·32
29	LXIV	M	76	410	2·86	0·454	6·98	1·76	1·82	0·450	4·44	1·095	15·73	3·942	38·37	9·62
32	LXV	M	76	496	3·76	0·950	7·58	1·91	1·98	0·498	3·99	1·003	20·06	5·120	40·44	10·32
33	LXVI	F	76	512	3·47	0·876	6·78	1·71	2·12	0·536	4·14	1·045	24·18	6·212	47·23	12·13
36	LXVII	M	76	410	3·46	0·880	8·44	2·15	1·89	0·480	4·61	1·171	15·82	3·980	38·59	9·71
38	LXVIII	F	76	400	3·33	0·838	8·33	2·10	1·72	0·430	4·30	1·075	12·72	3·210	31·80	8·03
40	LXIX	F	76	463	3·66	0·902	7·90	1·95	1·98	0·496	4·28	1·071	19·52	4·662	42·16	10·07
42	LXX	F	52	400	3·28	0·861	8·20	2·15	1·88	0·472	4·70	1·180	18·92	4·718	47·30	11·80
45	LXXI	M	52	365	2·80	0·730	7·67	2·00	1·82	0·420	4·99	1·151	12·68	3·126	34·74	8·56
Mean				436			7·20	1·70			4·23	1·01			38·60	9·00
Standard Deviation							0·663	0·305			0·329	0·158			5·533	1·69
Probable Error of Mean							±0·10	±0·05			±0·05	±0·02			±0·84	±0·26

SERIES III.

Group 3 (continued).

Ligation of Vessels of Left Kidney.

Number of Animal.	Number of Litter.	Sex.	Time after Operation.	Body Weight at Death.	Weight of Organs in Grammes with their Ratios (× 1,000) to Body Weight in Crammes.											
					Kidney.				Heart.				Liver.			
					Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).		Weight.		Ratio (× 1,000).	
					Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.	Wet.	Dry.
2	LII	F	76	423	2·82	0·571	6·67	1·35	1·42	0·290	3·36	0·69	17·45	3·518	41·25	8·32
3	LIII	M	76	476	3·04	0·612	6·39	1·29	1·90	0·391	3·99	0·82	16·82	3·416	35·34	7·72
5	LIV	M	76	482	3·15	0·646	6·54	1·34	1·79	0·366	3·71	0·76	19·00	3·887	39·42	8·06
7	LIV	M	76	431	3·07	0·622	7·12	1·45	1·72	0·358	3·99	0·83	16·32	3·478	37·87	8·07
9	LV	F	76	416	2·75	0·568	6·61	1·37	1·46	0·299	3·51	0·72	16·42	3·467	39·47	8·33
11	LVI	M	76	432	2·72	0·561	6·29	1·30	1·50	0·316	3·47	0·73	16·05	3·421	37·15	7·92
13	LVII	F	76	415	2·85	0·581	6·87	1·40	1·47	0·299	3·55	0·72	14·28	2·966	34·41	7·15
15	LVIII	F	76	463	3·35	0·678	7·24	1·46	1·67	0·342	3·61	0·74	19·18	3·921	41·49	8·47
17	LIX	M	76	487	3·46	0·698	7·10	1·44	2·01	0·420	4·13	0·86	21·65	4·472	44·45	9·15
18	LIX	F	76	418	2·60	0·671	6·22	1·61	1·76	0·452	4·21	1·08	14·85	3·916	35·53	9·30
19	LX	M	76	465	3·45	0·872	7·42	1·88	2·05	0·521	4·41	1·12	19·15	4·916	41·18	10·57
22	LXI	M	76	459	3·34	0·842	7·28	1·84	2·05	0·526	4·47	1·15	18·78	4·991	40·91	10·87
24	LXII	F	76	427	3·02	0·761	7·07	1·78	1·92	0·488	4·49	1·15	14·59	3·682	34·17	8·62
25	LXIII	F	76	415	3·02	0·761	7·28	1·84	1·88	0·491	4·53	1·18	16·28	4·081	39·23	9·83
27	LXIII	F	76	395	2·87	0·722	7·27	1·83	1·82	0·458	4·62	1·16	16·28	4·081	41·21	10·33
28	LXIV	M	76	400	2·53	0·635	6·33	1·83	1·78	0·445	4·45	1·16	13·82	3·456	34·55	8·97
30	LXV	F	76	403	3·06	0·766	7·59	1·90	1·86	0·466	4·62	1·16	16·40	4·202	40·69	10·42
34	LXVI	M	76	487	3·12	0·782	6·41	1·60	2·02	0·516	4·19	1·06	16·55	4·162	33·98	8·55
35	LXVII	M	76	456	3·38	0·822	7·71	1·80	1·98	0·472	4·34	1·06	22·00	5·612	48·24	12·32
41	LXIX	F	76	498	3·65	0·920	7·33	1·85	2·12	0·530	4·26	1·06	26·12	6·700	52·45	13·45
44	LXXI	F	52	380	2·88	0·720	7·58	1·90	1·68	0·420	4·42	1·11	11·72	2·812	30·84	7·40
Mean				439			6·96	1·62			4.12	0.97			39·14	9·21
Standard Deviation							0·491	0·226			0·430	0·176			5·676	1·55
Probable Error of Mean							±0·07	±0·03			±0·06	±0·03			±0·84	±0·23

THE EFFECT OF TEMPERATURE ON THE UNEQUAL INTAKE OF THE IONS OF SALTS BY PLANTS

by

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> "If Donnan's theory of membrane equilibria furnishes the mathematical and quantitative basis for a theory of colloidal behaviour of the proteins, as the writer believes it does, it may be predicted that this theory will become one of the foundations on which modern physiology will have to rest."—Loeb (24).

INTRODUCTION.

It has been known for some considerable time that, when a plant tissue or root-system is immersed in a solution of an electrolyte, the two ions of the electrolyte are absorbed in different proportions, and in the case of neither ion does absorption necessarily proceed to an equilibrium position characterized by equality of concentration within and without. For this conclusion, indeed, there is now an accumulation of evidence: Meurer (28), Ruhland (42), and in particular Stiles (44), found that the ions of salts were absorbed in unequal proportions by isolated storage tissues; and Pantanelli (34), Redfern (37), and Hoagland (20) observed the same phenomenon in the case of absorption by entire plants. Unequal internal and external equilibrium concentrations of ions have also been found in the case of *Nitella* by Hoagland and Davis (21), and by Osterhout (32) in the case of *Valonia*. Not without interest in this connection, too, is the heaping up of the coloured ion of certain dyes in plant cells, first observed by Pfeffer (36), and the comparatively small absorption in the case of others (Collander, 8). Many other observations of a like nature could be quoted, details of some of which may be obtained from the review of Stiles (43).

These results point to the existence of some physico-chemical mechanism controlling absorption, whereby some ions are trapped and accumulated in the protoplast and others are largely excluded: a mechanism of this kind must be very fundamental in the life-processes of the cell, and it is likely to be intimately associated with many phenomena, such as antagonism, selective absorption from the soil, and, indeed, all the manifold interrelationships between the plant and its nutrient medium.

Of the nature of this mechanism we have much to learn; for in all the above-quoted work there is no suggestion of a definite rule operating in the absorption processes; in some cases the anion was absorbed in excess, in other cases the cation. It has been shown by Stiles (44), however, that the equilibrium position in the intake of an ion is dependent on its concentration: with decreasing external concentration relatively more salt was found to be absorbed, the equilibrium being given approximately by the equation:

$$y = kc^m,$$

where y is the final internal concentration, c the final external concentration, and k and m are constants. This equation is that which has been held to be characteristic of adsorption processes, and has consequently led Stiles to believe that adsorption may be a governing force in the absorption of the ions of salts by plant cells.

Apart from this, however, practically no investigation has been made of the influence of external factors on the equilibrium position in the absorption of ions by plants; and the present paper has been devoted to the study of the influence of temperature on the equilibria of ionic intake, in the hope of making a further contribution to our knowledge of the underlying mechanism of this process. The temperature factor was specially selected for study, since it has received perhaps least previous consideration; our only knowledge of its effect being a few observations by Miss Redfern (38), which indicated that, in the intake of methylene blue by carrot tissue, absorption decreased with rise in temperature. No investigation has been made of the effect of this factor on the unequal intake of the two ions of a single electrolyte, to which attention has been particularly directed in the present work.

It is a pleasure to record my gratitude to Professor A. J. Ewart, F.R.S., who has done much to facilitate the performance of this investigation; I am indebted also to Professor T. Brailsford Robertson, of the University of Adelaide, for kind suggestions and criticism. The expenses of the experimental work were defrayed in part by a grant from the research funds of the University.

EXPERIMENTAL.

TECHNIQUE.

The method adopted in studying absorption in the present investigation was that which has been brought into use by Stiles (44). The parenchymatous tissue from the central region of the root of the carrot was used in the form of circular discs 1 mm. in thickness and 17·5 mm. in diameter. These discs were prepared by means of a cork-borer and a sliding microtome, and were then washed for twelve hours in running tap-water, followed by successive changes of distilled water extending over several hours. This process was carried out in order to remove material from the cut surface cells and also as much as possible such substances as might tend at first to diffuse out from the tissue; it served also to allow the tissue to attain a uniform and maximum degree of turgidity.

After washing, the discs were drained on filter paper in sets of forty, each set then being placed in a flask containing 100 cc. of the solution of which the absorption was to be measured. In any series of solutions used in an experiment, each set of discs was taken from the same original batch of carrots, as it was found that different batches showed a certain amount of variation in the results given, although the main conclusions to be drawn were not modified. In order to ensure that the tissue remained healthy, Stiles weighed the sets of discs before and after the experiment, and concluded by the constancy of the weight whether turgidity had been maintained. It was found, however, that death of the tissue, resulting in complete loss of turgor, could readily be observed by the flabby nature of the discs in such condition, so that the weighing was dispensed with in later experiments; and, moreover, loss in weight, owing to osmotic effects resulting from immersion in the experimental solution, may occur without affecting the vitality of the cells.

The process of absorption was followed by removing samples at intervals and analyzing them, until it was seen by the flattening of the curve of absorption in relation to time that equilibrium had been reached. On the removal of each sample, discs were taken out in such number as to keep constant the ratio of the number of discs to the volume of the liquid. In the tables of results only the equilibrium values for absorption have been given.

It was found that equilibrium was generally attained when about eighty hours had elapsed from the commencement of the experiment. This was a much longer period than was required in the case of Stiles' experiments, but in his case the preparations were agitated continuously during the process of absorption, while in the present instance mixing could be effected only at intervals of some hours.

A number of sets of discs immersed in the experimental solution were prepared for each experiment, and these were kept at various temperatures, ranging from 4–30°C. These temperatures were obtained in a special laboratory artificially kept at 10°C. Temperatures above this value were maintained in ovens controlled by gas-mercury-toluene thermostats, and lower temperatures were obtained in a refrigerator, the unvarying temperature of the room rendering all values constant to within 1°C.

Difficulty was experienced at first in keeping the samples above 20°C. free from infection, and for this reason sterilization of the laboratory and all apparatus and solutions became necessary. It was found also that above 30° the tissue tended to die before the equilibrium position was reached, the discs losing turgor and becoming quite flabby.

The hydrogen-ion concentration of the various solutions was determined at the beginning and end of most of the experiments, to ensure that changing reaction might not be in part responsible for the results obtained. These estimations were carried out by the indicator method, using standard buffer solutions for comparison.

Experiments were conducted, using the dyes methylene blue and erythrosin A in ·005% aqueous solution, and the salts ammonium chloride, ammonium sulphate, potassium chloride, and calcium nitrate in ·1M. concentration.

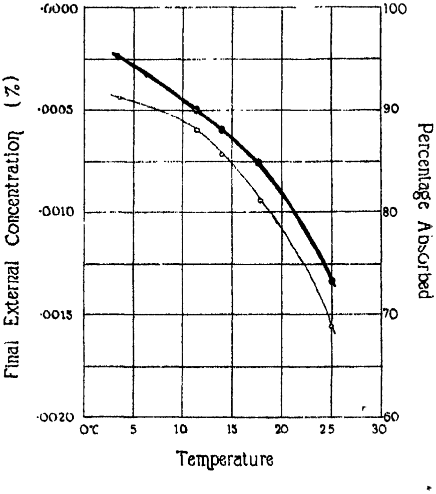

Figure 1. Curves showing the relation of the final external concentration to temperature in the absorption of ·005% methylene blue in two typical experiments. The upper curve is constructed from the results given in Table 1.

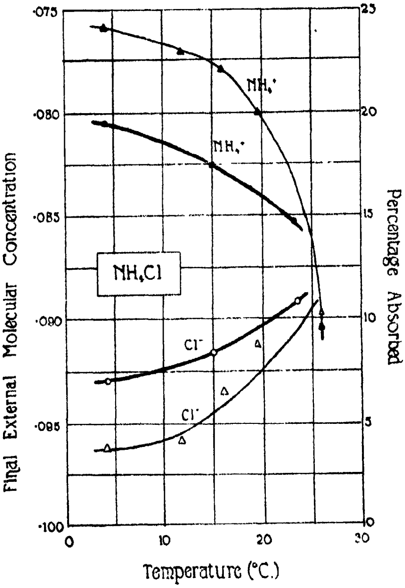

Figure 2. Curves showing the relation of the final external concentration of $NH_4{}^{\cdot}$ and Cl' to temperature in the absorption of ·1M ammonium chloride in two typical experiments. The heavy curves are constructed from the results in Table 2.

TABLE 1.

Equilibrium Position in the Intake of 005% Methylene Blue at Various Temperatures.

Temperature.	Initial Ext. Concn.	Final Ext. Concn.
25°C.	·0050%	·00133%
18	”	·00074
14	”	·00058
11	”	·00050
3	”	·00023

RESULTS.

The Intake of Methylene Blue.

The investigation was commenced with a study of the absorption of methylene blue, in order to compare the results obtained with those of Redfern (38) previously mentioned. The absorption was measured in ·005% aqueous solution of the dye, at temperatures of 25°, 18°, 14°, 11°, and 3°C., the methylene blue being estimated colorimetrically. In each case the experiment was repeated a number of times with remarkably uniform results, which as a whole exhibited the same trend as those of Redfern, although they differed numerically to a considerable extent. In Table 1 are given the results of a typical experiment with methylene blue, these being also expressed graphically in figure 1.

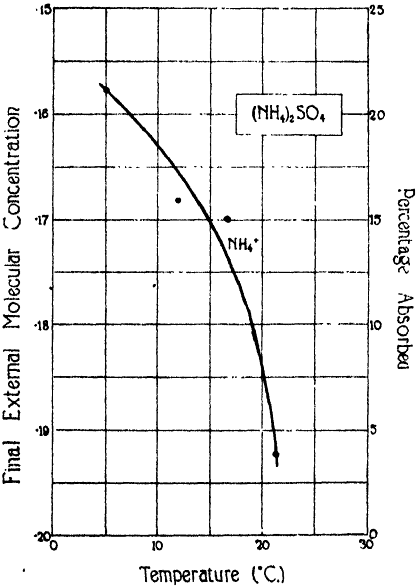

Figure 3. Curve showing the relation of the final external concentration of $NH_4^{\cdot}$ to temperature in the absorption of ·1M $(NH_4)_2SO_4$. Constructed from results given in Table 3.

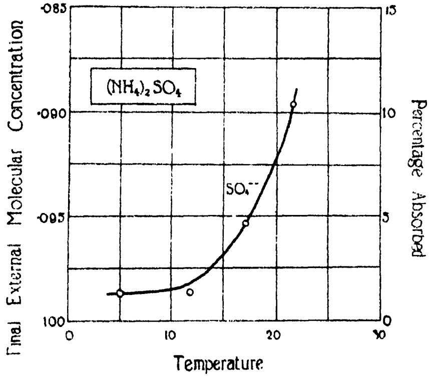

Figure 4. Curve showing the relation of the final external concentration of SO_4'' to temperature in the absorption of ·1M $(NH_4)_2SO_4$. Constructed from results given in Table 3.

The Intake of Erythrosin.

Experiments were conducted in a precisely similar manner to those with methylene blue, using a ·005% aqueous solution of erythrosin A. The amount of absorption, however, was small, and no marked temperature effect was obtained; moreover, accurate estimation was rendered difficult on account of changes which took place in the colour of the dye during the course of the experiment. For these reasons the use of erythrosin was discontinued, more especially as the main interest of the investigation lay in the use of salts, of which both ions could be readily estimated, rather than in the use of dyes.

The Intake of Ammonium Chloride.

The absorption of ·1M ammonium chloride was observed under conditions similar to those of the previous experiments on dyes. Ammonium was estimated colorimetrically after Nesslerization, while chlorine was estimated by the process of Volhardt. In Table 2 are given the results of a typical experiment. The relation of the final external concentration to temperature is also expressed graphically in figure 2.

The Intake of Ammonium Sulphate.

The previous experiments were repeated, using ·1M ammonium sulphate. Ammonium was estimated as before, and the sulphate radicle was precipitated and weighed as barium sulphate. Typical results are given in Table 3 and figures 3 and 4.

Table 2.

Equilibrium Position in the Intake of the Ions of ·1M NH_4Cl *at Various Temperatures.*

	$NH_4^{\cdot}$		Cl'				
Temp.	Init. ext. concn.	Final ext. concn.	Init. ext. concn.	Final ext. concn.	$NH_4^{\cdot} \times Cl'$	Original P_H of solution.	Final P_H of solution.
4°C.	·100M	·0805M	·100M	·0930M	·00748	6·7	5·8
15	,,	·0825	,,	·0916	·00755	6·7	5·7
23	,,	·0855	,,	·0892	·00758	6·7	6·0

The Intake of Potassium Chloride.

The absorption of ·1M potassium chloride was studied in a manner similar to that used for the previous salts. Potassium was estimated by precipitating with sodium cobaltinitrite (Macallum's reagent, 25), according to a method similar to that originally devised by Aidie and Wood (1). Five cubic centimetre samples of the experimental solution were used, and to each of these 10 cc. of the reagent were added. After standing for twelve hours the precipitate was filtered in tared filters, washed, and dried at 125°C. to constant weight. As a microchemical process the accuracy of this method has been subjected to some criticism, but with the large quantity of precipitate used in the present estimations satisfactory results were readily obtained. In carrying out these analyses I received much assistance from my father, the late Dr. J. M. Petrie, of the University of Sydney, who had made this method a subject of special study. The chlorine, as in previous cases, was estimated by Volhardt's method. Results of a typical experiment are give in Table 4 and figure 5.

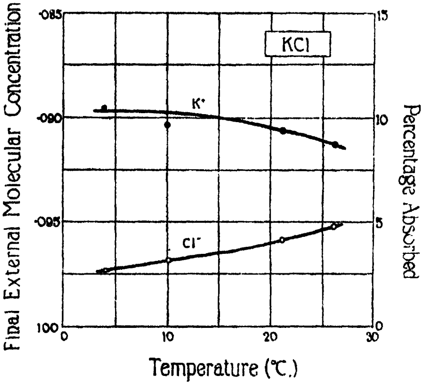

Figure 5. Curves showing the relation of the final external concentration of $K^{\cdot}$ and Cl' to temperature in the absorption of ·1M potassium chloride. Constructed from results given in Table 4.

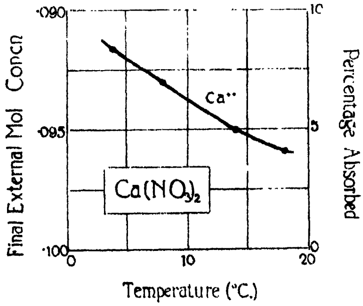

Figure 6. Curve showing the relation of the final external concentration of $Ca^{\cdot\cdot}$ to temperature in the absorption of ·1M $Ca(NO_3)_2$. Constructed from results given in Table 5.

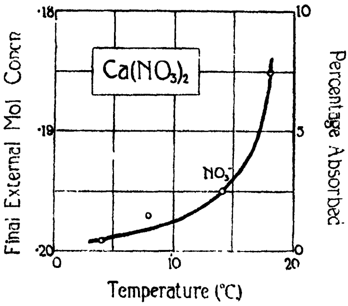

Figure 7. Curve showing the relation of the final external concentration of NO_3' to temperature in the absorption of $Ca(NO_3)_2$. Constructed from results given in Table 5.

The Intake of Calcium Nitrate.

Similar experiments were carried out on the absorption of ·1M calcium nitrate. Calcium was estimated by precipitating with oxalic acid and titrating the excess acid, while the nitrate was estimated colorimetrically after conversion to ammonium picrate. The results of a typical experiment are given in Table 5 and figures 6 and 7.

DISCUSSION.

THE RESULTS ANALYSED.

The results of the experiments carried out with methylene blue indicate that with increasing temperature there is a progressive fall in the equilibrium intake of the dye. The same tendency was shown in the few observations made on this subject by Miss Redfern, as has already been mentioned.

The curves showing the relation of the final external concentration to temperature (figure 1) indicate that, especially at the lower temperatures, a very large proportion of the dye is absorbed into the tissue. In this connection it is interesting to bear in mind that methylene blue is a basic dye, or in other words, the results obtained pertain to the absorption of the positive ion only; and, while this accumulation within the tissue characterizes the basic dye, the absorption of the acid dye, erythrosin, or rather its acid radicle, which is the coloured portion, was only slight. These facts are in agreement with Pfeffer's observations (36), more recently confirmed by Redfern (38)[1], that while plant cells rapidly absorb basic dyes, acid dyes are in general absorbed only to a slight extent or not at all. They are also in agreement with Ruhland's earlier adherence to this view (40, 41), and with Overton's conclusion that only dyes soluble in lipoid substances enter the cell (33), since on the whole basic dyes are soluble in lipoids, while acid dyes are not.

These experiments with dyes, however, are of a subsidiary nature: the main interest of the investigation lies in the results obtained with such electrolytes as ammonium and potassium chlorides, ammonium sulphate and calcium nitrate, wherein both cation and anion were estimated. If the curves in the various figures are examined, it will be seen that a single general principle is operating in them all, independent of the particular salt used. It is moreover apparent that the effect of temperature on the equilibrium concentration is remarkably different for the two ions of the salt: as with methylene blue, the equilibrium intake of the cation falls as the temperature rises; but, on the other hand, the amount of the anion absorbed increases with the temperature. From the variety of salts used, it would appear that these are fundamental properties to be associated with monovalent and divalent cations and anions in general; and it will be the purpose of the remaining pages to consider how these properties may be interpreted.

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[1] Miss Redfern in this paper falls into the error of supposing that aniline blue is a basic dye; in reality it is acidic, and it is noteworthy that Miss Redfern finds that it behaves in a manner similar to the other acid dyes which she used.

TABLE 3.

Equilibrium Position in the Intake of the Ions of $\cdot 1M\ (NH_4)_2SO_4$ *at Various Temperatures.*

	$NH_4^{\cdot}$		SO_4''		
Temp.	Init. ext. concn.	Final ext. concn.	Init. ext. concn.	Final ext. concn.	$(NH_4^{\cdot})^2 \times (SO_4'')$
5°C.	·200M	·158M	·100M	·0985M	·0033
12	,,	·167	,,	·0985	·0028
17	,,	·170	,,	·0953	·0028
21·5	,,	·192	,,	·0897	·0025

THE SIGNIFICANCE OF THE ABSORPTION FORMULA.

In the Introduction to this paper it was mentioned that in the absorption of an ion Stiles had found the equilibrium position to be dependant on the concentration, in accordance with the equation

$$y = kc^m.$$

This equation was found by Redfern (38) to hold also in the case of the absorption of the coloured radicles of dyes by carrot tissue.

The importance of Stiles' discovery is that this equation is identical with the adsorption isotherm of Freundlich (16), which connects the amount of the so-called process of "adsorption" with the equilibrium concentration. This equation has been held by many authorities in the past to be characteristic of the physical process of surface aggregation as contrasted with actual chemical combination; and, in the light of the above results, as was mentioned in the Introduction, Stiles has suggested that the ions taken in by the tissue are "adsorbed" at the surface of some cell constituent or constituents, probably colloidal in nature. In support of this view some authorities might cite also the few observations of Redfern indicating a decrease in the percentage absorption of dyes with increase of temperature, a property likewise held to be "characteristic of adsorption processes as distinct from chemical combinations" (38) (see also Bayliss, 4, p. 61). It may be remarked here, however, that the results of the present investigation, while similar to those of Redfern in the case of methylene blue and cations in general, are not so in the case of anions, and, if a negative temperature coefficient is held to be a characteristic property of adsorption, then this process is not a direct cause for the intake of these ions.

At the same time Stiles has pointed out that chemical combination of the absorbed salt with a cell constituent is unlikely, since in such terms it would not be possible to explain the fact, manifested in his results, that the ratio of the final internal to the final external equilibrium concentration, which he terms the absorption ratio, is less than unity in the case of the higher concentrations of the solution. "For," as he states, "if the salt is capable of combining with a cell constituent, it must be concluded that it is capable of entering the cell, and it would then be supposed that, after all the particular cell constituent combining with the salt was utilized, absorption of the salt would continue until there was equality of concentration of the salt on the two sides of the cell-membranes. The chemical combination view, therefore, also involves the assumption that the salt itself, or the compound formed by its action within the cell, renders the cell membranes impermeable to the salt, so that absorption stops before enough of the salt is taken up to bring about equality of concentration on the two sides of the membrane. The very regular relation between concentration and absorption, however, militates against the view that a cell-membrane is rendered impermeable in high concentrations of the salt, while low concentrations have no such action."

The chemical combination view, however, does not necessarily involve such assumptions as Stiles would suppose, for it has been shown that many purely chemical reactions in a heterogeneous medium conform to an equation of the Freundlich type, a frequently quoted example being the union between haemoglobin and oxygen. Michaelis and Rona (31) have also shown that the absorption of basic dyes by filter paper is not a process of physical adsorption, as was formerly supposed (Bayliss, 4), but is due to a chemical substitution of the basic radicle of silicates in the paper by the base of the dye. Loeb (24) has shown that adsorption by proteins is a stoichiometric replacement of hydrogen or basic ions according to the state of the protein with reference to its isoelectric point. Fisher (15) in studying the phenomena of absorption in soils has demonstrated that the Freundlich equation applies with an equal degree of accuracy to many reactions that are not adsorptions, and he points out also that, as the exponent *m* is altered, a series of curves is obtained to one of which nearly any set of experimental points can be fitted with the desired degree of accuracy: adsorption in soils is to be explained as a base exchange in the salts of the weak colloidal acids which are now known to be among their most important constituents (5, 6, 19). The Wilsons (47) have rejected the adsorption formula as applying to colloidal problems, and have adopted a purely chemical interpretation; and its unsoundness as a criterion of any particular type of reaction has been urged by many other writers, e.g., Arrhenius (2), Bradfield (5), Gordon (17), Langmuir (22), Robertson (39). There seems every reason to believe, as Mathews (27) has suggested, that adsorption of most substances from solution, including acids, bases, ions, salts, dyes, etc., is due to chemical forces, the adsorbed substance forming a true chemical union with the surface molecules of the adsorbent.

TABLE 4.

Equilibrium Position in the Intake of the Ions of ·1M KCl *at Various Temperatures.*

	$K^{\cdot}$		Cl'				
Temp.	Init. ext. concn.	Final ext. concn.	Init. ext. concn.	Final ext. concn.	$K^{\cdot} \times Cl'$	Original P_H of solution.	Final P_H of solution.
4°C.	·100M	·0896M	·100M	·0973M	·0087	6·6	5·8
10	,,	·0904	,,	·0969	·0088	6·6	6·0
21	,,	·0906	,,	·0958	·0087	6·6	5·8
26	,,	·0912	,,	·0953	·0087	6·6	5·8

It appears, then, unnecessary to suppose, with Stiles, that the chemical combination theory is incompatible with the adsorption equation: in a process of base exchange, for example, the extent to which replacement takes place must vary exponentially with the equilibrium concentration of the entering cation, and so conform with the general types of Freundlich's equation, although at the same time the replacement is a chemical one. And judging by analogy with the works that have been quoted, a chemical interpretation for the absorption of ions by plant cells is perhaps more likely than one relying upon purely physical phenomena. We have digressed thus far to arrive at this conclusion, since it is in terms of a chemical combination theory that a possible interpretation of the present results seems to lie.

BASE EXCHANGE IN COLLOIDAL ACIDS.

From the results of various investigators it seems well established that, in the unequal absorption of the constituent ions of a salt, the excess of cation over anion absorbed is replaced in the external solution by the diffusion out from the tissue of an equivalent amount of other positively charged ions. Thus Stiles (44) has shown that in the absorption of sodium chloride by carrot tissue the excess $Na^{\cdot}$ absorbed is replaced by $Ca^{\cdot\cdot}$, $K^{\cdot}$ and $Mg^{\cdot\cdot}$; Redfern (37) likewise found that in the absorption of calcium chloride by living plants, the excess of $Ca^{\cdot\cdot}$ ions was replaced by $Mg^{\cdot\cdot}$ and $K^{\cdot}$; and Stocklasa and his collaborators (45) found a similar replacement by $Ca^{\cdot\cdot}$, $Mg^{\cdot\cdot}$ and $Na^{\cdot}$ in the case of the absorption of $Al^{\cdot\cdot\cdot}$ and $Fe^{\cdot\cdot\cdot}$ ions.

These results leave no doubt that the process of cation absorption is one of base exchange. Now in our discussion of adsorption, it has been realized that many reactions characterized by the adsorption formula are in reality cases of chemical exchange of bases in colloidal salts; and one is thus led to consider the possibility of a similar interpretation for the phenomenon at present under consideration. Such a conception would be reconciled with Stiles' results indicating the régime of the adsorption formula; and, moreover, in the light of our previous conclusions upon the significance of this formula, its very applicability to electrolytic absorption by plant tissue points, if to anything, to a colloidal base exchange such as is here suggested.

The existence of such a negative colloidal radicle as would operate in a process of base exchange of this kind is no assumption. It is well known that protoplasm contains many colloids, numbers of which must be in the form of negative ions. Among these are obviously the proteins: the chief protein in carrot tissue is a globulin of which the isoelectric point is at P_H 4·3 (7); and, since the normal reaction of the tissue is about P_H 6·0 (35) and the final external P_H in the experiments described in the present paper was never as low as 4·3, the protein in the cell-protoplasm must exist in the form of colloidal anions, with which base exchange could readily take place. One may mention also the phosphatides which, as Levene (23) has pointed out, are amphoteric like the proteins, and may play a fundamental rôle in the metabolic processes of the cell (cf. 9, 18). Perhaps of more significance, however, on account of their greater profusion in plant protoplasm (cf. Macdougal, 26), are the carbohydrates: many of these substances are known to be dissociated as weak acids or their salts (Euler, 12, 13; Michaelis and Rona, 29, 30) and it seems highly probable that the colloidal polysaccharides occurring in plant protoplasm will behave in the same way: Fairbrother and Mastin (14) have, in fact, shown that this is so in the case of sugar, and have also shown that base exchange with this colloidal acid salt takes place with ease. We have not yet the evidence to suggest which of these substances might be concerned in the present problem; but it is clear that colloidal anions, with which replaceable basic ions must be associated, occur in abundance in plant protoplasm.

TABLE 5.

Equilibrium Position in the Intake of the Ions of ·1M $Ca(NO_3)_2$ *at Various Temperatures.*

	$Ca^{\cdot\cdot}$		NO_3'				
Temp.	Init. ext. concn.	Final ext. concn.	Init. ext. concn.	Final ext. concn.	$Ca^{\cdot\cdot} \times (NO_3')^2$	Original P_H of solution.	Final P_H of solution.
4°C.	·100M	·0917M	·200M	·199M	·0036	10·0	6·6
8	,,	·0930	,,	·197	·0036	10·0	6·6
14	,,	·0950	,,	·195	·0036	10·0	6·4
18	,,	·0959	,,	·185	·0033	10·0	6·4

THE DONNAN EQUILIBRIUM.

The system as we conceive it in the plant cell may now be regarded fundamentally as one of two solutions separated by a semi-permeable membrane. The internal solution is that of the colloidal salts in the protoplasm, separated from the external experimental solution by the plasma-membrane of the cell. To all the crystalloidal ions in the system this membrane is to be regarded as permeable, while on the contrary it is impermeable to the colloidal anions within.

A system such as this is one in which we might well suppose that a Donnan equilibrium would operate. It has been shown by Donnan (10, 11) that, when two solutions of electrolytes are separated by a membrane which is impermeable to one of the ions of one of the electrolytes, the diffusible ions proceed to an equilibrium at which they are unequally distributed on the two sides of the membrane. At this equilibrium, also, the product of the concentrations of each pair of oppositely charged ions is the same on either side; and these products are constant whether only a single electrolyte is concerned or whether there is a mixture of a number.

This equilibrium condition can be expressed by the equation

$$x^2 = y(y + z),$$

where x is the concentration of the positive and of the negative ions on one side of the membrane, y is the concentration of the positive and of the negative free crystalloidal ions on the side containing the indiffusible ion, and z is the concentration of crystalloidal ions in combination with the indiffusible ion.

The membrane equilibrium can be expressed also by the equation

$$ax = iy,$$

where a and x are the concentrations of any pair of oppositely charged ions in the one solution, and i and y are the total concentrations of the same pair of ions on the other side of the membrane.

If one of the ions, e.g. the negative one (x,y) is divalent, these equations become

$$x^3 = y^2(y + z)$$

and

$$a^2x = i^2y.$$

It is no new departure to suggest the application of the Donnan equilibrium to living protoplasm: Donnan himself stressed the importance of its potential biological applications on account of the existence in living organisms of just such semi-permeable membranes as the theory requires. This has been further upheld by Loeb (24); and more recently several investigators have applied the Donnan theory to the distribution of electrolytes in blood (3, 46, 48). It will be the purpose of the concluding pages to show that, if we assume this theory to hold in the case of plant cells also, it provides an interpretation for the results obtained in the present inquiry.

THE RESULTS INTERPRETED.

In the case of the non-diffusible ion being negative, as has been concluded to be the case in the present instance, then of any pair of crystalloidal ions in the system, the Donnan theory requires that the external concentration of the cation should be less than the internal, and that the reverse should be the case with the anion. Starting then with equal concentrations of ions outside, when the equilibrium position has been reached the external concentration of cation should be less than that of the anion, while the reverse should hold for the internal concentrations: stated more simply, i will be greater than y, and a will be less than x.

It will be observed that this relationship of a to x holds in all the experiments recorded in the present work: and it is obvious that the relationship of i and y must hold also, since the internal concentrations, although not capable of estimation experimentally, must be in inverse ratio to the external concentrations.

The results show, however, that with increase of temperature the value of a rises and that of x falls, wherefore the excess of i over y must decrease. Such a decrease would be explained if the effect of rising temperature were such as to cause a decrease in the value of z, the concentration of the indiffusible negative ion.

Now such a decrease is what might well be expected to follow rise in temperature. Many colloidal suspensions, e.g. powdered gelatine, tend to pass into true solution under such conditions; and, passing into solution, might then be able to diffuse out through the plasma-membrane and so become less concentrated within the cell: or their exosmosis might occur at higher temperatures purely as a result of the increasing permeability of the plasma-membrane, which is known to be proportional to the temperature. In the case of at least one of the colloidal anions of the protoplasm, viz., the phosphatides, it is definitely known that outward diffusion occurs in increasing amount with elevation of temperature (9).

In any case, as Professor T. Brailsford Robertson, of the University of Adelaide, was kind enough to point out to me, it may be taken as a general rule that rising temperature decreases the combining capacity of tissue colloids for bases, which would be equivalent to a decrease in the value of z. I am indebted to Professor Robertson also for the suggestion that polymerization of the colloid with higher temperatures, as for example is known to take place on coagulation, might reduce its combining capacity for bases, and so reduce the concentration of colloidal anions.

If, then, we are justified in suggesting that the value of z falls with rising temperature, it follows from the Donnan equations that, since the total amount of the experimental electrolyte in the system does not alter, not only should ax equal iy at any particular temperature, but the values of these products must remain the same at all temperatures; and the same should hold for the two sides of the equation as modified for difference in the valency of either of the ions.

It is unfortunately impracticable to determine experimentally whether the external and internal ionic products are equal, on account of the impossibility of determining the internal concentrations of the ions. Stiles (44), it is true, has calculated empirical values for these concentrations, based on the difference between the initial and final concentrations. Values obtained in this way, however, would have no significance in the present case, since Stiles has to assume an empirical and constant estimate for the volume of the solvent containing the electrolyte in the tissue. It is thus obvious that Stiles' calculations of the internal concentration are not only empirical but may not even be proportionally correct, since the volume of internal solvent is certain to change from the assumed constant value with alteration of temperature.

So far as the external concentrations are concerned, however, the results of this investigation have shown that their products in any given experiment have a constant value independent of the temperature, which, as we have seen, is what the Donnan theory requires.

On the whole there seems much in support of an interpretation of the absorption of the ions of electrolytes by plants as a process of base exchange in colloidal acids or their salts, such process being governed by the Donnan equilibrium: this conception has at all events provided an interpretation for the results of this inquiry which does not seem to be readily forthcoming in other terms.

It might be urged, however, that since we are adopting a theory based on chemical foundations, it should be possible to demonstrate that stoichiometric relationships exist, and that only the valency of the ions concerned should produce variations in the curves obtained; whereas not only do the curves differ somewhat for various salts, but even for the absorption of the same salt by different batches of tissue. Such stoichiometric relationships, however, could be demonstrated only by the use of material in a precisely similar state for each experiment, which in living organisms it is impossible to obtain. Every batch of tissue must contain somewhat different amounts of colloids, and inevitable fluctuations in the internal hydrogen-ion concentration must cause differences in their degree of dissociation, and hence a further alteration in the value of z in the Donnan equation; except in the case of the proteins, moreover, the degree of dissociation of the colloids may alter with different bases[2]; and variations in the previous history of the tissue may lead to dissimilar concentrations of the ions to be absorbed already being present in the tissue, which again would result in absorption proceeding to a different extent, and would further help to explain inaccuracies in the empirical determinations of the internal concentrations. Only the relative shapes of the curves and the constancy of the external products can be regarded as unfailing criteria under all conditions of the material; and to these criteria the results of the various experiments hold true.

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[2] Non-ionized molecules can be considered as if wholly removed by precipitation; it is particularly when partial precipitation takes place that Fisher (15) has shown the adsorption formula to apply to chemical combinations.

It is not the purpose of this paper to put forward the Donnan equilibrium and stoichiometric base exchange as being the controlling factors in the unequal intake of the ions of electrolytes by living plants. It merely suggests that, if these phenomena indeed take place, as is not impossible, they will provide a satisfactory interpretation of our present knowledge of this process of ionic intake which no other theory seems to give; should it ultimately be possible by deeper and more extensive researches to establish the régime of these factors as unquestioned, then it is perhaps no rash assumption to suggest that its significance will equal that of any former discovery in the nutrient relationships of the living cell.

SUMMARY.

The experimental portion of this paper comprises a study of the effect of temperature on the absorption of the ions of single electrolytes by isolated plant tissue.

It is found that with increasing temperature the amount of cation absorbed falls and the amount of anion absorbed increases; and that these alterations take place proportionally, resulting in a constancy of the products of the two ions in the external solution.

Reasons are given for the probability that the absorption of ions by plant cells is a process of base exchange in dissociated colloidal acids or their salts; and it is suggested that a Donnan equilibrium may be set up between the colloidal anions of the protoplasm on the one hand and the external medium on the other.

Such an hypothesis provides a satisfactory explanation for the results obtained on the effect of temperature on the unequal absorption of the ions of electrolytes, an explanation which does not seem to offer itself in terms of any other theory.

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ON THE ORIENTATION OF MITOCHONDRIA IN THE SURFACE CYTOPLASM OF INFUSORIANS

by

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(Submitted for publication 12th July, 1927.)

INTRODUCTION.

It will be noticed, when surface sections of infusorians are carefully examined, following a mitochondrial technique, that the mitochondria present a striking orientation at the surface of these organisms. This interesting orientation has been observed by several authors, and the object of this paper is to investigate and suggest an interpretation for this impressive phenomenon.

MATERIAL AND METHODS.

In order to carry out this investigation two common types of infusorians were employed, namely, *Paramoecium* and *Nyctotherus cordiformis.* The former organisms were obtained from a culture grown in a solution of malted milk (1 gr. per 1,000 cc.), while the latter were obtained from the rectal contents of starved frogs *(Hyla aurea)*, which occasionally swarm with *Nyctotherus cordiformis.*

The following mitochondrial technique was employed during this investigation: All material was fixed in either a Flemming solution without acetic, or else in a Champy fluid, and afterwards washed out in running water for several hours following a 24 hours fixation.

These small organisms were then concentrated by means of centrifuging, brought up through the alcohols, cleared, and finally embedded *en masse* in paraffin, and afterwards sectioned 3–5μ in thickness. The sections were then stained by Heidenhain's iron-haematoxylin process, and occasionally counterstained in eosin. Examination of cut and stained sections with high magnifications through the mid-nuclear region of both these organisms show numerous small, rod-shaped, bacteria-like bodies, together with a lesser number of small spherical individuals, scattered irregularly throughout the cytoplasm; a denser accumulation of granular bodies around the outer surface of the nuclear membrane, such as has already been described in a previous paper (1), may also be observed. When, however, surface sections of these protozoa are examined, it will be seen quite clearly that these rod-like bodies present a striking orientation in the surface cytoplasm of these cells.

That these bacteria-like bodies are mitochondria, and not symbiotic or parasitic organisms, is clearly shown by their subsequent reaction to certain stains and fixatives. Bacteria may be distinguished from rod-like mitochondria by exposing them for several hours in strong alcohol. Bacteria after such treatment stain up very sharply after staining with the Heidenhain process, while mitochondria, on the other hand, are rendered non-stainable by iron-haematoxylin after quite a short fixation.

In fact, any fixative containing large proportions of either acetic acid or alcohol render mitochondria non-stainable. It is also well known that the great majority of successful bacterial fixatives contain large proportions of alcohol.

All material used in this investigation was finally tested by the alcohol process; and, moreover, these cytoplasmic inclusions, present in both organisms, coloured up a bluish-green when stained *intra vitam* with a janus green B. solution (1 : 8,000), and the results obtained show clearly that these rod-shaped, bacterial-like bodies are true mitochondria, reacting in a manner similar to mitochondria present in other animal and plant cells.

OBSERVATIONS AND DISCUSSION.

When *Nyctotherus* and *Paramoecium* in longitudinal sections, after staining with Heidenhain's iron-haematoxylin process, are examined, it is observed that

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EXPLANATION OF PLATE.

All figures (except figure 3) are drawn from material fixed in a Flemming solution without acetic acid, or else in a Champy fixative. All sections were then stained with Heidenhain's iron-haematoxylin, and occasionally counter-stained in eosin.

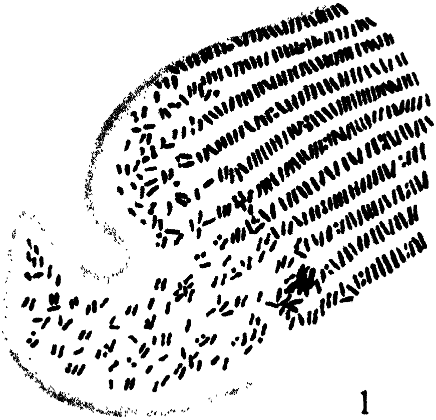
Figure 1. Oblique surface section of an adult Nyctotherus, showing orientation of mitochondria. Note how the rod-like mitochondria are arranged one behind the other, exhibiting a more or less transverse polarity to the axis of the cell. Observe also the irregularity of mitochondria in the deeper parts of the organism. Several mitochondria may be seen in the dumb-bell shaped condition.

Figure 2. Surface section of Paramoecium, demonstrating the arrangement of mitochondria in the surface cytoplasm. Note how the mitochondria occur one behind the other, presenting a longitudinal polarity to the infusorian.

Figure 3. Transverse section through the outer region of an infusorian, demonstrating the position of the myoneme threads. Note the corrugations in the cytoplasm between each intervening myoneme thread. (After Butschli.)

Figure 4. Longitudinal section through the mid region of the nucleus of Nyctotherus, showing mitochondria scattered irregularly throughout the cytoplasm, together with large granular masses of mitochondria lying round the outer surface of the nuclear membrane. Micronucleus and apparently empty food vacuoles clearly visible.

Figure 5. Longitudinal section of an adult Paramoecium cut through middle of cell, showing mitochondria aggregated around outer surface of meganucleus. Large numbers of mitochondria are also seen scattered throughout the protoplasm. Micronucleus and food vacuole containing mitochondria are clearly seen.



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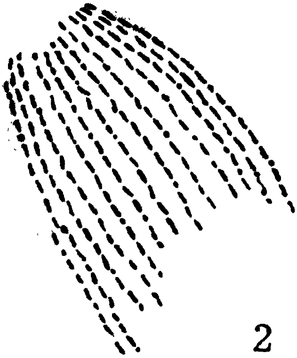

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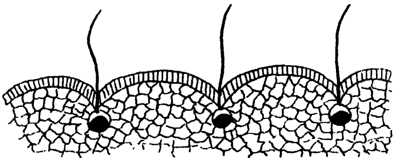

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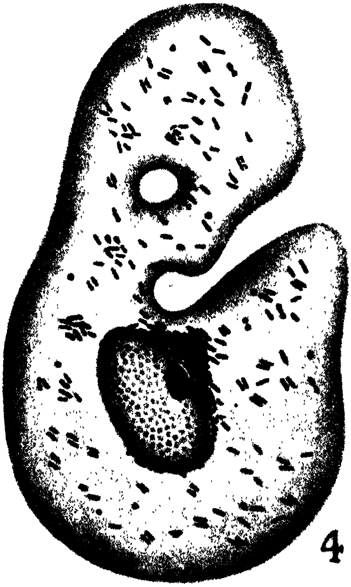

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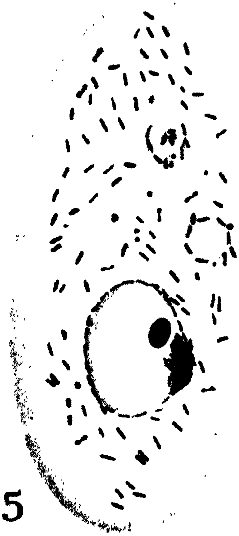

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the cytoplasm of these organisms contains a large number of rod-shaped mitochondria, together with a smaller percentage of spheroidal bodies scattered throughout the protoplasm, the rounded individuals arising from the rod-shaped mitochondria by means of a transverse binary fission.

If, however, a section cut through the middle of the meganucleus be examined, it will be observed that the mitochondria are scattered in fairly large numbers, irregularly throughout the cytoplasm, while lying quite close to, and apparently in intimate contact with, the outer surface of the nucleus, may be observed a granular mass of mitochondria (see figure 4).

While examining the surface sections of these organisms I further observed that the rod-like mitochondria present in the surface cytoplasm exhibit a striking orientation; and at the same time a closer observation revealed the mitochondria in greater abundance in this region of the infusorian than in the centre. (See figure 1.)

A separate examination of *Nyctotherus* and *Paramoecium* demonstrates a marked difference in the arrangement of the orientation of the mitochondria in the surface cytoplasm of these two organisms. (See figures 1 and 2.)

If a surface section of *Nyctotherus* showing the orientation of mitochondria be observed under magnifications, it will be seen that the rod-like mitochondria are arranged one behind the other in longitudinal rows, each single mitochondrium exhibiting a polarity more or less transverse to the axis of the cell; while on the other hands an examination of the orientation of the mitochondria in *Paramoecium* shows that the rod-like mitochondria are arranged one behind the other also in longitudinal rows, but in this case the mitochondria present a longitudinal polarity to the axis of the infusorian.

A more detailed study of the surface sections of both these organisms reveals the presence of myoneme threads, which show up quite clearly after the cell has been treated with the ordinary mitochondrial fixatives. These myoneme threads run parallel to and beneath the rows of cilia, and according to Minchin (2) are a characteristic feature of the ciliate infusoria.

Owing to the presence of these myoneme threads, which, as has already been stated, run in longitudinal rows parallel to the axis of the cell, the surface cytoplasm is thrown into corrugations or folds. (See figure 3.)

Now in a previous paper (1) I have pointed out that the surface accumulation of mitochondria in cells may be interpreted in the light of their phosphatidal composition; and the same theory may be brought forward to explain how they come to lie round the inner surface of the cell membrane of the organisms at present under discussion. They would naturally accumulate in the corrugations, and the intervening myoneme threads would cause their segregation into distinct longitudinal rows.

It has been observed that in different infusorians the number and proximity of these myoneme threads varies. In *Paramoecium*, for example, they are so closely adjacent to one another that the mitochondria are only able to lie end to end, thus giving rise to a longitudinal orientation. In *Nyctotherus*, on the contrary, the distance between each myoneme thread is greater, thereby allowing the rod-like mitochondria to lie side by side in a position transverse to the axis of the cell.

Thus it is strongly suggestive that the difference in the orientation in these two infusorians may be due to differences in the proximity of these myoneme threads to one another.

SUMMARY.

The orientation of mitochondria in the surface cytoplasm of infusorians may be correlated with their phosphatidal nature, together with the presence of myoneme threads.

This investigation was carried out during the tenure of a Government research scholarship in Zoology at the University of Melbourne.

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by

O. W. TIEGS

CORRECTIONS.

The following corrections should be made to:

A Comparison of the Utilization of Nucleic Acids of
Animal and Vegetable Origin

by

T. Brailsford Robertson, C. Stanton Hicks, and Hedley R. Marston.

Page 128, line 30, for *against* read *again*.
Page 149, line 19, for *successfully* read *successively*.

The following corrections should be made to:

The Effect of Temperature on the Unequal Intake of the
Ions of Salts by Plants

by

Arthur H. K. Petrie.

For *Arthur K. H. Petrie* read *Arthur H. K. Petrie* throughout.
Page 177, line below table, for *Absorption* read *Adsorption*.
Page 180, line 22, for *sugar* read *agar*.

A CRITICAL REVIEW OF THE EVIDENCE ON WHICH IS BASED THE THEORY OF DISCONTINUOUS SYNAPSES IN THE SPINAL CORD

by

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(Submitted for publication 21st July, 1927.)

Despite the researches of several investigators, who have upheld the theory of a protoplasmic continuity at the neurone junctions within the central nervous system, the doctrine of the discontinuous synapse, embracing the body of the nerve cell, or ramifying among its dendrites, has gained an almost general acceptance during the last thirty years. This has been in the main due to the great authority of its main supporter, Ramon y Cajal. In several recent papers I have pointed out that there is but little evidence in favour of this doctrine, but that on the contrary there occurs within the spinal cord a true neurone continuity, not, indeed, a continuity through a superficial Golgi network, as described by Bethe, but a continuity due to the penetration of the collaterals into the dendrites of the nerve cell. The purpose of the present paper is to review the evidence on which the synapse theory is based, and at the same time to discuss the theory of continuity in the form in which it has been presented by other investigators.

Much of the criticism here given of the synapse theory I have already made in previous papers (23, 24, 25); the evidence for the existence of discontinuous synapses within the spinal cord has been so widely accepted, however, and the doctrine is now so generally held, that it seems worth while to take stock of this evidence once more, the more so since a recent reviewer, Peterfi (22), has remarked that the theory of continuity, in the form in which I have stated it, will gain but little acceptance in the face of so much direct evidence for the theory of contact synapses. In this discussion I hope to show that the evidence on which the contact theory is based is, so far as the spinal cord is concerned, extraordinarily scanty.

It will be well to point out at once that the synapse theory is a histological and not a physiological conception. The term synapse was first used by Michael Foster and Sherrington (13) in 1897 for the branching terminations of neurones, described by Cajal as ramifying amongst the dendrites or embracing the body of a nerve cell σὺν and ἅπτω (clasp). It is true that the term has entered largely into the literature of a reflex physiology, and that many of the characteristics of reflex action have been attributed to its action.[1] Nevertheless, it is clear that it is entirely a histological idea, and reflex physiology might just as clearly be formulated in terms of any other integrating mechanism in the nerve cells. Consequently, while a study of the neurone junction must have a profound bearing upon the theory of the integrative action of the nerve cell, it cannot play the same part in the theory of the integrative action of the nervous system as a whole.

The outstanding feature of the conception of the reflex arc in the histology of twenty years ago was a protoplasmic discontinuity between its cellular components. The theory of a contact relationship of motor neurone and skeletal muscle fibre has now been replaced by the knowledge that the nerve termination is definitely hypolemmal; even in the case of plain muscle, where Cajal, Retzius, Arnstein, and others found a free termination of nerve endings *between* the muscle fibres, Boeke (6) and Lawrentjew (18) have clearly demonstrated an intraprotoplasmic termination of the nerve fibres. So in the relation of a sense cell to its afferent neurone, Boeke has shown by a series of striking drawings, that the end ramifications of the nerve fibres penetrate into the substance of the receptive cell, frequently terminating near its nucleus (cornea, foliate, and circumvallate papillae, Eimer's organ in the mole, Merkel's corpuscles, taste buds, and Grandry corpuscles); he concludes that the free endings observed by Cajal,[2] Retzius, and others, were due to incomplete staining (5). When protoplasmic continuity can thus be demonstrated at the two ends of the reflex arc, it does not seem probable that discontinuity should exist in its middle. Our present attitude towards the question is inconsistent; thus, while those physiologists who maintain the theory of the myogenic origin of rhythmical contraction of plain muscle tissue look to the protoplasmic bridges between the muscle fibres to transmit the stimulus from fibre to fibre, they do not hesitate to accept the theory of a break in the arc in that very region where conduction might be expected to be most highly specialized, namely, in the central nervous system.

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[1] While the histological bases of most of the component phenomena of nerve integration are quite unknown, there is one, namely fatigue, whose presence can readily be detected. Fatigue of the reflex arc is accompanied by a disappearance of Nissl granules within the nerve cell (Gustav Mann). This observation seems to show quite clearly that in the vertebrate spinal cord, at any rate, fatigue is a phenomenon of nerve cell activity, and accords but ill with the theory that it is a phenomenon of the synaptic membrane situated in close contact with the outer surface of the nerve cell. The observation suggests most strongly that the whole of the integrative processes of the reflex arc occur within the body of the nerve cell, and argues strongly against their occurrence outside the cell. This conclusion is certainly in accord with independent histological evidence described in this paper.

[2] Cajal maintained an interprotoplasmic ending of sensory fibres as late as 1921 (see Cajal, 9). Boeke's results are, however, quite decisive on this point.

Before the introduction of the special methods of Golgi, Ehrlich, and Cajal, the spinal cord was considered, on the basis of Gerlach's observations with carmine stained preparations (1870) to be a diffuse network, the network being formed by an extensive anastomosis of the branching dendrites with one another. The axis cylinders, which had been discovered by Deiters, were regarded as arising by the fusion of outgrowths from the cells of this network and from the network itself.

With the discovery by Golgi (about 1873) of the silver chromate impregnation process, the presence of long conducting fibres within the grey matter was for the first time clearly observed. Golgi failed to confirm the observation of Gerlach of a diffuse network formed by the fusion of dendrites; he found that the dendrites, undergoing extensive branching, ended blindly, and the cell axons were formed as outgrowths, one from each nerve cell. Nevertheless, Golgi still adhered to the theory of a continuous protoplasmic network within the nervous system; he believed that collaterals from the axon of one neurone formed a continuous network with similar collaterals from another neurone, a conclusion which neither Kölliker nor Forel nor Ramon y Cajal could confirm. The diffuse network, he believed, could be specially clearly demonstrated in the spinal cord and cerebellum; but while still maintaining that a continuity between the branching collaterals of two neurones was demonstrable, he held that the close contact between the two systems was enough to transfer the stimulus from one to the other. The diffuse nervous net of Golgi seems to be the dense mass of fine fibrils that are seen pervading the grey matter in very completely stained Golgi and reduced silver preparations.

The main criticism that may be raised against Golgi's conception of the diffuse network is that it is not directly demonstrable. When we observe, in very completely stained preparations (see, for example, figure 4), a dense meshwork of fibrils in the grey matter, it is obviously a matter of mere speculation to regard this as a continuous network; occasional cases of fusion of fibrils may be readily considered as one of the many artefacts observable in Golgi preparations. In reduced silver preparations in which very complete staining has occurred, no indication of a network can be detected. Nevertheless, Golgi appears to have held this view, though in a modified form, even till his death. This attitude towards the synapse theory of the man to whom we are so largely indebted for our present knowledge of the minute structure of the central nervous system shows, at any rate, that the theory of synapses is not based upon such readily demonstrable evidence as we usually suppose. For a statement of Golgi's views his address delivered at Stockholm in 1906 may be referred to (15). This theory of the diffuse network was also upheld by Nansen (21), from studies on the nerve cord of numerous invertebrates, and in addition of Amphioxus and Myxine.

Golgi's theory of the diffuse nervous network was speedily replaced through the work of Cajal, van Gehuchten, Kölliker, and others, by the theory of the discontinuous synapse. By means of the methylene blue process of Ehrlich, and especially of the silver chromate method of Golgi, Cajal was enabled to trace nerve fibres with their finest branches often over incredible distances. These fibres appeared to be of two different kinds: there were those which could be observed to form a pericellular nest on the body of other nerve cells, such as the endings of fibres on the cells of sympathetic ganglia in the frog, or the pericellular terminations of fibres of the acoustic nerve on the cells of the trapezoid nucleus (Held), or the ending of the basket fibres on the Purkinje cells of the cerebellum; and in this group might also be classed the extraordinary neurone junctions occurring in the olfactory glomeruli. The second type of neurone junction was one in which the fibre underwent extensive branching in the grey matter, and as good examples of this type might be taken the branching collaterals in the cerebral cortex and in the spinal cord.[3] It is with this second type that we are especially concerned here. Of the first type it need only be said that the observations of Boeke (5) on the staining reactions of the terminations of nerve fibres seem to show that Cajal's technique in this matter has been very faulty; while, for example, Cajal describes free terminations of nerves upon the surface of the Purkinje cells, Bielschowsky and Wolff (4) find that delicate neurofibrils pass across from these terminations and penetrate the substance of the cell. Marui (20) is able to observe the same thing in the giant Mauthner cell of the fishes' brain. Anybody who has been able to observe how inadequate is the Golgi or Ehrlich method for revealing the intra-protoplasmic termination of sympathetic fibres in plain muscle cells, will lay but little stress upon preparations which show blind terminations of fibres in the central nervous system.

In preparations of the spinal cord, especially of young animals, made by the Golgi technique, the nerve cells have the appearance of enormously branching cells, whose dendrites in some cases may ramify over a very large area of the grey matter contained in the section. As Golgi first stated, and as Cajal and most others have since affirmed, these branching dendrites eventually end free in the grey matter. In some Golgi preparations a considerable number of such nerve cells are to be seen, but relatively few collaterals are stained. It is a curious feature of this method that at other times great numbers of collaterals may stain, while the nerve cells in turn remain unstained. In his earlier work (7) Cajal could not satisfy himself concerning the endings of these fibres. But subsequently he concluded, and his views were confirmed by Kölliker and others, that they terminated freely in the grey matter. Occasionally one also obtains Golgi preparations in which a dense meshwork of nerve fibres and also numerous nerve cells have stained. On account of the complexity of the preparations, however, such material is declared by Cajal to be unsuitable for demonstrating the neurone junctions. Cajal's remark is to a certain extent valid; it is certainly extraordinarily difficult to disentangle this dense meshwork with the microscope. The incompletely stained preparations are, however, even less suitable for revealing the relation of collateral to nerve cell; for if only one or the other of these is rendered visible, it is purely a matter of speculation what the relation of one to the other will be. Cajal's general conclusion was that, since in some preparations the branching nerve cells were not visibly connected with nerve fibres (the dendrite branches ending freely), and since in other preparations the branching collaterals always ended freely in the grey matter, there must therefore be a break in the chain, and the neurone junction must be one of contact and not of protoplasmic continuity.

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[3] It is possible that there is a fundamental difference between these two types of ending. The first type is characterized by the fact that one neurone, without forming collaterals, terminates by an end-ramification upon the body or dendrites of another cell, and a marked convergence of neurones upon this cell does not occur; the second type is characterized by an extensive collateral formation, one neurone terminating on numerous other cells, each of these cells having at the same time numerous collaterals from other neurones ending upon it. While the latter type is specialized for performing integration between excitatory and inhibitory impulses reaching it from different nerves, it does not seem possible to ascribe this property to the first group (the basket fibres of the cerebellum may be an exception to this).

It is clear from these remarks that the theory of synapses, built up as it is from evidence obtained by Golgi preparations, rests upon indirect evidence; in some preparations branching nerve fibres are found to have free endings, while the nerve cells are unstained; in others free nerve cells are found, but branching nerve fibres remain unstained; and it is inferred that the latter end upon the former, the peculiarly selective staining being attributed to a break in the neurone chain.

Although Cajal has given innumerable such illustrations of branching collaterals stained by the Golgi method, ending apparently free in the grey matter, and numerous other illustrations of nerve cells also entirely free, yet, so far as I am aware, he has never observed stained branching collaterals terminating in close contact with the body or dendrites of simultaneously stained nerve cells. For example, if figure 108 of Cajal's book be examined, there is no direct evidence to show that the branching collaterals that enter the grey matter terminate upon any of the stained or upon any of the unstained nerve cells or their dendrites. So far as I am aware there is only one instance in which such a relationship was believed to have been directly demonstrated, namely, that shown in figure 110 of Cajal's book, drawn from a methylene blue preparation, a method which Cajal admits is unsuitable for staining the vertebrate central nervous system. This constitutes the entire direct histological evidence on which the theory of the discontinuous synapse in the spinal cord was originally founded. In addition to these observations there was a considerable mass of indirect evidence in support of the theory. Cajal summarizes this evidence as follows:

(a) The demonstration by His that the neuroblasts of the developing cord are morphologically independent, with the implication that such independence is maintained in the fully developed animal. This evidence loses its force when we consider that a similar independence between a muscle fibre and its motor nerve, observable in the embryo, no longer occurs in the fully developed animal, the motor ending being definitely hypolemmal.

(b) Primary degeneration of a nerve fibre on severing it from the body of the nerve cell does not extend on to the next neurone of the chain. But again it may be pointed out that neither in the case of the motor nerves does it extend beyond the end of the nerve; the ensuing muscular atrophy is a secondary atrophy, and occurs long after the primary degeneration of the nerve fibre is over.

(c) With the methylene blue technique Retzius failed to detect a neurone continuity in the invertebrate nerve cord, an argument which again loses its force if we bear in mind Boeke's warning that this stain cannot always be relied upon to demonstrate the finest nerve fibres (see Boeke, 5).

We see, therefore, that the indirect evidence brought forward by Cajal in support of his doctrine of neurone discontinuity, namely, embryological evidence, evidence obtained from degeneration experiments, and evidence obtained by analogy with invertebrate tissue, is invalid. Let us now examine the direct histological evidence itself.

Silver chromate preparations have led, in the hands of Cajal, Golgi, Kölliker, van Gehuchten, and others, to two conclusions already stated, namely, (1) An independence of the nerve cells, with free endings of dendrites; and (2) A free termination of collaterals that enter the grey matter. Of the latter Kölliker (17) says: "All the collaterals, without exception, end, as it seems, in the same way, namely, with fine end ramifications, which remind us of the endings in ordinary motor end-plates. These ramifications consist of innumerable short branchlets of the finest varicose fibrils, which closely envelop the nerve cells and end usually with fine knobs." In numerous successful Golgi preparations of the nerve cord of newly-born rats and mice I have made the following observations: (1) The dendrites of the nerve cells terminate either by being cut short as they emerge from the section, or they may show an apparently genuine ending. (2) The collaterals end either by being cut short as they emerge from the section, or their ending may be apparently genuine, and these fibres, after undergoing branching, may end in a varicosity, though much more frequently such varicosities are not found. Illustrations of the endings of fibres are given in figures 1 and 4. In such preparations, therefore, the observation of Cajal, and of the other upholders of the neurone theory, can undoubtedly be confirmed. It seems to me, however, that the observation loses its entire point when we consider *that innumerable instances may be found where the nerve fibres on being traced backwards towards or into the grey matter, are found also suddenly to end* *without emerging from the plane of the section.* In many Golgi preparations, of course, the collaterals can be observed arising directly as lateral outgrowths from the longitudinal fibres of the white matter. But in many highly successful Golgi preparations the relationship cannot be demonstrated. In such preparations the stain does not reveal any connection between the collaterals and the longitudinally running axons from which they have arisen; the observation can

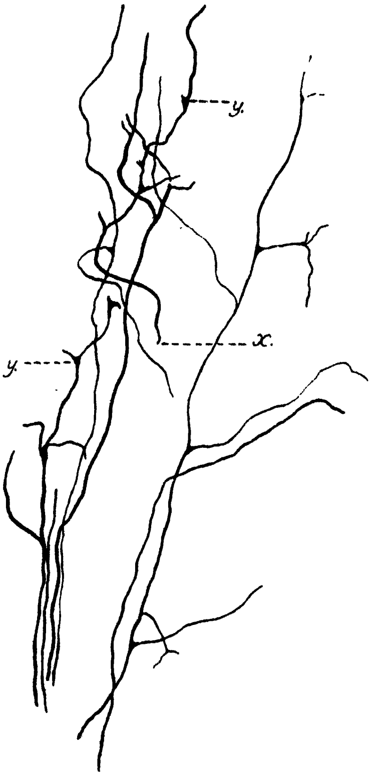

Figure 1. Branching collaterals from spinal cord of new-born mouse, as revealed by the Golgi method. The collaterals, which are taken from a region of the cord adjacent to the central canal, are shown undergoing secondary branching. Most of the collaterals cannot be traced outwards into the white matter, because they are cut short as they emerge from the plane of the section. But at **x** is shown a collateral which, on being traced outwards, ends, not because it is cut short, but because the staining has ceased (the figure cannot, of course, reveal this). The terminations of the finer branches of the collaterals are also not due, in this preparation, to their emerging from the section; it would be unjustifiable to consider them genuine endings or as providing evidence in favour of Cajal's doctrine of terminations by free "end-knobs," since (1) the fibres do not terminate in "end-knobs," and since (2) at some places (indicated by **y**) the staining is obviously incomplete. The illustration is intended to show that even in highly successful Golgi preparations cessation of the staining reaction does not necessarily imply a termination of the nerve fibre.

be made with the greatest ease, and no confusion has arisen with collaterals which have been cut short as they emerge from the plane of the section. We are accustomed to believe that the Golgi technique is capable of revealing collaterals or single nerve cells and all their dendrites in their entirety; these observations show on the contrary that the belief is entirely unjustified. If we once grant, as it seems we must, that the "reazione nera" of Golgi may suddenly cease along the length of a nerve fibre, then we will have to admit that the observation of a free ending of collaterals and of dendrites, upon which Cajal has built the contact theory, cannot be accepted.[4] I need only draw attention here to the recent remarks of Boeke (5) on the extreme difficulty of rendering visible especially the fine terminal parts of delicate nerve fibres; in the case of plain muscle (as has been shown by the use of the Golgi method in Cajal's own hands) it is inadequate.

We have seen, therefore, that the theory of discontinuous synapses in the cord was built up originally on four distinct masses of evidence: (1) embryological, (2) experimental, (3) analogy with invertebrate tissue, and (4) histological; and each of these bodies of evidence we now find is invalid.

The diffuse nerve net of Golgi was, however, so impossible to demonstrate satisfactorily in the cord that the doctrine of a free termination of collaterals on the surface of nerve cells, as first enunciated by Cajal, became generally adopted. On the basis of Cajal's observation Waldeyer in 1891 formulated the neurone theory, a theory which maintains a morphological and physiological independence of neurones in the central nervous system, the neurones being related to one another by contact and not by protoplasmic continuity.

With the subsequent discovery of the reduced silver methods for staining nervous tissue, evidence of a more direct kind was claimed by Cajal for the contact theory; cases were described of nerve fibres ending by "terminal buttons" on the nerve cells, and direct observations of discontinuity in the reflex chain seemed to be apparent. These terminal buttons were held to be identical with similar structures observed by Held (16) and by Auerbach (2), but again we note that these "terminal buttons" were believed by their discoverers to be definitely fused with the body or dendrites of the nerve cell. They appear, indeed, to be structures first seen by Deiters in maceration preparations, and described by him in his famous posthumous work in 1865. Deiters held that the nerve cells were provided with a single large axon, and numerous very delicate axons which grew out from the body, and dendrites of the nerve cell from minute conical papillae. This free "secondary system of axons" can readily be demonstrated in Golgi preparations, but was considered by Golgi and Cajal to represent merely a branching of the dendrite. I believe, on the contrary that the original idea of Deiters was much nearer the truth than the subsequent opinion of Golgi and Cajal; it can be shown, in fact, that the "secondary system of axons" which Deiters described are in reality the fine endings of collaterals that have grown *into* the nerve cell, and not out from it, as Deiters thought, and that they are the endings of collaterals which Cajal could not observe. It seems to me that the erroneous interpretation of Golgi and Cajal has arisen because the Golgi method is unable to reveal the true nature of the fine fibrils that appear to grow out from the cells and dendrites (but in reality grow *into* them), for it stains the whole cell intensely black, and cannot reveal that these fine fibres are really continued into the nerve cell as its neurofibrils (see figure 4). It is the reduced silver method that can reveal the true nature of these fibres; but it was on the evidence of Golgi preparations that the theory of discontinuity was founded, and the results of the new technique of twenty years later do not seem to have been able to shake it.

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[4] Whilst the body of most of the nerve cells in successful Golgi preparations is perfectly free from nerve fibres, one frequently encounters nerve cells with several nerve fibres closely adjacent to the body of the nerve cell. Occasionally one may even observe structures that have the appearance of an end-foot applied to the surface of the cell. Lest it be urged that these fibres are the actual terminations of collaterals, it may be pointed out that there is no evidence in favour of this; some of these fibres are definitely connected with the cell (and are probably collaterals that have penetrated it); others appear to pass over the cell, but their passage cannot be rendered visible, on account of the deep black staining of the cell; fragments of other fibres are also visible, and they may occasionally be seen to "end" in small or larger varicosities, which sometimes lie close to the body of the cell, at other times a considerable distance from it. When we recollect the extraordinary crudeness of Golgi preparations when studied under very high magnifications, we will cease to attribute any significance to these structures; in all probability they are fragments of incompletely stained, delicate fibrils, such as may be observed often in any situation in the grey matter.

With this new technique, however, Cajal claimed, as already stated, to be able to demonstrate the terminal buttons at the ends of the fine collaterals, lying in close contact with the body of the nerve cells. My own results, obtained by this method, are profoundly different; I have not been able to demonstrate the terminal "end-buttons," but on the contrary find that the collaterals grow into the dendrites of the nerve cells, and become continuous with its neurofibrils, an observation which Cajal does not seem ever to have made.

I shall discuss the "terminal buttons" first. By means of the reduced silver technique Cajal has rendered visible what he holds to be the actual terminations of the collaterals, namely, minute cone-shaped swellings at the ends of the fine fibrils, applied closely by their flat surface, to the surface of the nerve cell. For an illustration, figure 112 of Cajal's book may be consulted. In numerous apparently very successful preparations of the nerve cord, stained by this method, I have failed to observe these structures, so that it is difficult to form an estimate of them. We notice in these "terminal buttons," however, that they have never been shown to be connected with undoubted nerve fibres. Can it be that Cajal was observing the endings of neuroglia fibres? It may be pointed out that neuroglia cells may often be seen in close connection with nerve cells, and the endings of neuroglia fibres upon blood vessels frequently, as Cajal himself has found, have this conical appearance. Or can it be, as one so often finds with adult tissue, that a shrinkage of the cell from its surrounding intercellular matrix draws the latter into fibrils? It must be admitted that it is extremely difficult to obtain an explanation of this figure of Cajal, if one has not been able to obtain similar results oneself; nevertheless, in my own preparations almost every nerve cell seems to exhibit so definite a neurone continuity, though of a very different type from that considered by Cajal, that I find it difficult to accept Cajal's figure as decisive. This much seems certain: that if Cajal is able to observe individual instances of terminal buttons applied to the surface of nerve cells, on the other hand the same method appears to reveal on almost every nerve cell the clearest evidence that the neurones penetrate into the substance of the cell. In any case, we observe in Cajal's own figure that the neurofibril staining has almost entirely failed; consequently he is not able to exclude the possibility of neurofibrils passing from his "end-buttons" into the substance of the nerve cell. One recalls the fact that Bielschowsky and Wolff actually describe such a neurofibril continuity in the terminal buttons which are applied to the Purkinje cells of the cerebellum, and that Donaggio observes the same in the nerve endings on the cells of the Trapezoid nucleus (11), while Cajal in both these situations finds nothing but discontinuity.

Contrary to the observation of Cajal, I find, as described in previous papers (23, 24, 25) that the collaterals which enter the grey matter undergo a considerable amount of branching, especially in the substance of Rolando, and that these collaterals then penetrate almost always into the dendrites of the nerve cells, being continued along these dendrites as the neurofibrils of the cell. Direct proof that the collaterals actually penetrate into the dendrites was at first not forthcoming; it is true that on every nerve cell that had been appropriately cut the neurofibrils of the cell could be observed to emerge from the dendrites and travel as naked neurofibrils in the substance of the grey matter, till they were cut short as they emerged from the plane of the section; and it is also true that the naked neurofibrils are indistinguishable in appearance from the branching collaterals that traverse the grey matter. But direct proof that the branching collaterals were the same as the naked neurofibrils was not at first available, until it was found possible to identify the neurofibrils with the branching collaterals, not by the fact that they arose as collaterals from what could be definitely identified as an axon of another cell, but because collaterals could be recognized in the grey matter by their manner of branching. A branching collateral generally exhibits a small triangular piece of protoplasm where the branch is formed, and the position of this minute piece of protoplasm reveals the forward direction of the collateral. Although, as previously reported (24), the method proved valueless for most of the collaterals which were examined (for even in sections 20μ in thickness they sooner or later emerge from the plane of the section), yet occasionally such a fibre could be followed to its destination, and it was then found to enter into the dendrite of another nerve cell. Whatever may be said concerning the "terminal buttons" of Cajal, it seems clear that if they really constitute a mode of neurone junction (leaving out of consideration the question whether they exhibit protoplasmic continuity with the cell or not), still the predominant and most easily demonstrated junction is one in which the axon collaterals, after undergoing branching in the grey matter, penetrate the dendrites of the nerve cell and form its neurofibrils. These neurofibrils then form a perinuclear network, as Cajal himself observed, and from this network a relatively small number of neurofibrils arise and enter the axon of the cell.

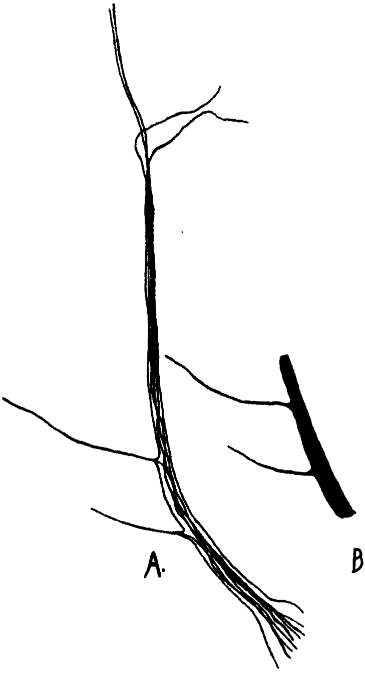

Figure 2. **A.** A dendrite from a spinal cord cell of rabbit, showing its neurofibril organization (reduced silver method). Six neurofibrils are clearly seen "emerging from" the dendrite. The point of "emergence" of two of the neurofibrils is marked by a minute outgrowth from the dendrite.

B. Diagram of portion of the same as it would appear in a Golgi preparation. This would be interpreted by Held as a case of two nerve fibres ending each by an "end-foot" upon the dendrite, the "end-foot" having fused with the dendrite.

If, as maintained above, this constitutes the predominant if not sole type of neurone junction in the cord, it may well be asked why Cajal failed to observe it. So far as I am aware, Cajal never observed naked neurofibrils emerging from the body or the dendrites of the nerve cells. None of his illustrations ever show neurofibrils emerging from the nerve cells and travelling as naked neurofibrils in the intercellular matrix of the grey matter. On the contrary, Cajal says of the neurofibrils of the nerve cell: In the dendrites "the bundles of neurofibrils thus become finer and finer, till only one remains, very thin, very pale, without varicosities in the core of the dendrite. These neurofibrils do not exist at all at the level of the dendritic spines; or, at least, if they exist they escape our sight and our methods, on account of their paleness and fineness. The neurofibrils which alone have remained in the core of the dendrite can in their turn ramify, as our observations on pyramidal cells have shown; they then end freely, and without a swelling at their ends." Cajal therefore seems to have failed to observe what is apparently the most characteristic feature of the neurofibrils, namely, that they may emerge from the nerve cells and travel for great distances free in the intercellular matrix of the grey matter. An illustration of this point is given in figure 2, drawn from one of many hundreds of cases observed. Numerous other illustrations will be found in my previous papers on this subject. Cajal's failure to make this observation is probably to be attributed to faulty preparations.

Edinger appears, however, to have known this fact, when on p. 25 of his book he remarks: "In addition to ganglion cells, neuroglia and nerve fibres, the grey substance is perhaps composed of numerous branches, which are formed from the nerve fibrils which enter it from the nerve fibres and the ganglion cells. For the vertebrates, the extent to which just this constitutes a component of the central grey matter is still unknown; we believe only that it exists." Edinger nevertheless adopts the conventional idea of the synapse.

In the above discussion it has been argued that while the doctrine of the discontinuous synapse was formulated almost entirely on the evidence of Golgi preparations, yet this method is not only incapable of revealing the penetration of collaterals into the body and dendrites of the nerve cells, but it is also inadequate to establish that very conclusion that has been drawn from it, namely, a neurone discontinuity in the region of the junction. It will now be pointed out that the evidence obtained from Golgi preparations leads to certain conclusions inconsistent with the theory of synapses ending on the body of the nerve cell or ramifying among its dendrites.

In diagrammatic representations of synapses, as they may be found in any text-book, we present a small nerve cell, with relatively short dendrites, ramifying among which are the terminal branching collaterals. The diagram is at first sight convincing enough; but examination of Golgi preparations frequently shows that structures which we call dendrites may frequently ramify over a very extensive region of the grey matter, in which case our diagram becomes difficult to accept. It might be urged against this argument that the small terminal ramification of the axon collateral ends upon a single dendrite only, or ramifies among branches of it; but this would be difficult to correlate with the fact that sometimes the dendrites that the Golgi method reveals, lie altogether outside the grey matter. For example, in figure 3, taken from Kölliker, it would be obviously impossible to urge a termination of collaterals upon the dendrites of the nerve cell, for the dendrites do not occur in the grey matter!

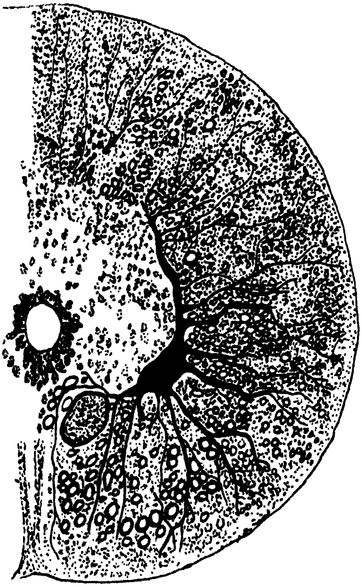

Figure 3. Transverse section of spinal cord of **Protopterus,** after Kölliker.

This might indeed almost be taken as evidence in favour of Golgi's view that nerve conduction was not a property of the dendrites, but that they had a nutritive function. The improbability of Golgi's view is, however, shown by the fact that the dendrites, as revealed by the Golgi method, are, except at their base, structures of extreme tenuity, and the extra free nutritive surface afforded by them must be negligible (see, for example, figure 4).

The objection against Cajal's thesis of the existence of terminal arborizations of collaterals upon the body of the nerve cells of the cord is that they have not been definitely demonstrated. In numerous successful Golgi preparations that I have studied, preparations in which both nerve cells and branching collaterals were sharply stained, I have been unable to find any evidence of nerve fibres converging upon the *body* of the nerve cells. In low-power examination, of course, such a condition may often be seen; we frequently observe a group of nerve cells, with collaterals in the surrounding grey matter showing a tendency to converge upon them. But when we study the same preparations under high magnifications, we cannot observe any convergence of nerve fibres upon the body of individual nerve cells. For instance, in figure 4 is shown a single nerve cell from the cord of a new-born rat. The cell is surrounded by a dense mass of fibres, but there is no indication that any of these fibres will terminate in a branching synapse on the body of the nerve cell.

There is one case in which Cajal has apparently *directly* observed the application of a terminal arborization to the body of a nerve cell, namely, the case shown in figure 110 of his book. It must be admitted that the figure is extremely

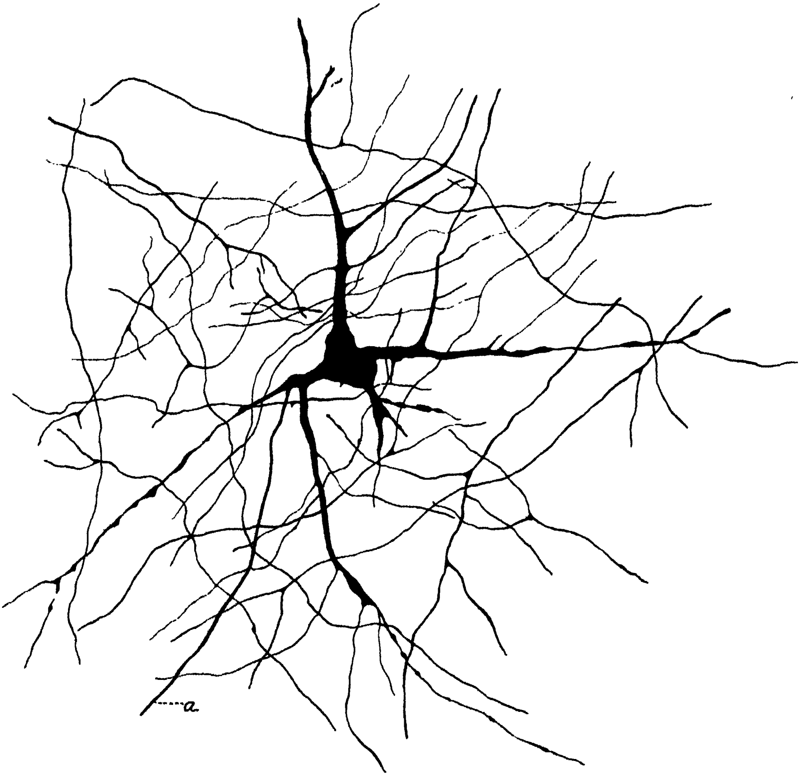

Figure 4. Nerve cell and adjacent nerve fibres from spinal cord of newly-born rat (Golgi method). The figure is intended to demonstrate the fact that, though innumerable fine, branched, and unbranched nerve fibres pass near the region of the nerve cell, none of these terminate by a branching ending (synapse) upon the body or dendrites of the cell, nor do any of them even show a tendency to converge upon the body of the nerve cell. On the other hand, three of the dendrites show clearly fine fibres, indistinguishable from the other nerve fibres, apparently growing out from their substance. But the Golgi method is unable to demonstrate that these fine fibres are continued into the dendrites as its neurofibrils. Only the reduced silver method can reveal the true nature of these fibres. Compare figure 2. Axon indicated at *a*.

convincing, even if it is drawn from a preparation made by the admittedly defective methylene blue technique. My own attempts to reproduce this result have always failed; with methylene blue, spinal cord tissue, as Retzius and others have stressed, stains extremely badly. I find that it presents all kinds of artefacts, among others, heavily vacuolated dendrites, which may assume the appearance of terminal arborizations embracing the nerve cell. But even this cannot account for Cajal's result. It may be pointed out, however, as any Golgi preparation will show, that the collaterals on entering the posterior horn of grey matter, may break into a brushwork of fine branches. In heavily stained preparations these branches, both in Golgi and in reduced silver preparations, may form a dense meshwork in the posterior horn. Can it be that the staining of the branches has ceased in such a manner as to give the appearance of a collateral ending by a branching termination on the body of the posterior horn cell? But whatever the interpretation of this figure of Cajal may be, even if we regard it as a genuine case of a collateral branching upon the body of a nerve cell, it cannot be taken to provide any evidence for nerve discontinuity.

We conclude, therefore, that while the predominant type of neurone junction in the spinal cord is one in which the collaterals penetrate the dendrites of the nerve cell, on the other hand a branching termination of neurones applied to the body of the nerve cell, while it is certainly very difficult to demonstrate, cannot yet definitely be disproved. But even if we admit this latter type of neurone junction to exist, there is no evidence to show that a true protoplasmic discontinuity occurs.

Lest it be suspected that the reduced silver and Golgi methods lead to different results, it may be pointed out that the results obtained by the two methods are quite consistent. While the reduced silver method demonstrates that the neurofibrils may leave the body of the nerve cell as naked neurofibrils in the intercellular region of the grey matter, the Golgi method, failing as it does to reveal any intracellular organization, merely stains the whole cell, with its emerging neurofibrils, deep black. The case is most forcibly illustrated by comparing the results of reduced silver, Golgi, and the average methylene blue preparations. The latter method does not as a rule stain neurofibrils or the great mass of delicate collaterals in the grey matter; on the other hand nerve cells and the dendrites are coloured dark blue. Now in these preparations the dendrites are relatively short, stout structures, do not branch very extensively, and are generally two or three times as long as the diameter of the cell. In successful reduced silver preparations the nerve cell presents the same appearance; in addition, however, the intracellular neurofibrils are sharply stained, and are found emerging from the dendrites and traversing the grey matter for relatively enormous distances, and only terminate when they pass out of the plane of the section. Finally, in Golgi preparations, the nerve cell and its neurofibrils are impregnated deep black, and we gain the impression of nerve cells with enormously long, delicate "dendrites" travelling far into the grey matter; the greater part of the delicate "dendrite," as the reduced silver method teaches us, is the naked neurofibrils that have emerged from the relatively short true dendrite (or rather, as I have argued, collaterals that have entered *into* the dendrites to become its neurofibrils), and it is only when they are cut short, or when the staining reaction fails along their length, that they appear to terminate (see figure 4).

The doctrine of continuity in the central nervous system has been developed especially by Apathy, Held, Bethe, and Bielschowsky; the theory of the diffuse nervous network of Golgi, as first enunciated, also involved the idea of protoplasmic continuity.

The theory of Apathy (1), as developed by that investigator from a study of the invertebrate nerve cord, maintains that neurofibrils emerge from the trunks of the sensory neurones, traverse the cord as naked neurofibrils, and, after having undergone a certain amount of anastomosis to form the "diffuses Elementargitter," penetrate the motor neurones. Within these they undergo partial anastomosis, and from the anastomosing network a small number (one in the leech) of neurofibrils enter the axon. In my own studies of the vertebrate nerve cord I have not found anything comparable with the Elementargitter, but except for this one point, which may in any case be one of secondary importance, my results are entirely identical with those of Apathy.

Bethe (3) conceived a system very similar to that of Apathy; he observed naked neurofibrils entering the body of the nerve cells, but maintained that these neurofibrils underwent extensive anastomosis on the surface of the cell to form the so-called Golgi net, which he considered to be homologous with Apathy's Elementargitter. This Golgi net, which is often to be observed in methylene blue preparations, was first observed by Golgi in 1893, and regarded by that investigator as a neurokeratin sheath with a skeletal function (14). I am entirely in agreement with Cajal that the Golgi net is a mere artefact, due to the coagulation of the pericellular lymph. If there is any doubt on this point, the figures in a paper by Marui may be consulted (20); it is true that this author holds the Golgi net to be nervous in function, but his figures surely render this interpretation inadmissible. Bethe does not seem to have observed the extensive penetration of the collaterals into the dendrites of the nerve cells, nor did he observe the perinuclear anastomosing network.

Held (16), while considering the Golgi net to be skeletal in function, described the existence of a so-called "pericellular nervous terminal net" on the surface of the nerve cell. He maintained that nerve fibres converge upon the body of the nerve cells, and undergo anastomosis here to form a continuous pericellular network, which has a different appearance from the Golgi net, and is to be looked upon as a different structure. To the argument that the nerve fibres are often found by the Golgi method, in direct connection with the Golgi network (with the inference that the latter is nervous in function), he replies that the stain has "jumped across" from the nerve fibre to the Golgi net. While this would admirably explain the connection between the nerve fibres and the non-nervous Golgi net, it would render Held's argument for the nervous nature of the "pericellular nervous terminal net" equally invalid.

Both the Golgi net and the pericellular nervous terminal net are, it seems to me, pure artefacts, due to coagulation of the pericellular lymph. I have certainly seen no trace of either in numerous very successful reduced silver preparations.

From the "pericellular terminal nervous net" arise, according to Held, the "end-feet," which bring the "pericellular terminal nervous net" into direct connection with the nerve cell. These end-feet exhibit protoplasmic continuity with the body of the nerve cell.

In his earlier papers Held described a loosening of the "axospongium" of the end-feet, as they fused with the protoplasm of the nerve cell, while at the same time the granules (neurosomes) in the end-feet merged into those of the nerve cell. In later papers he described in the case of the cells of the retina, the Purkinje cells, and the cells of the acoustic and trapezoid nuclei, a direct passage of neurofibrils from the end-feet into the protoplasm of the nerve cell.

It seems to me that the work of Held contains this one important element of truth, namely, that there is a passage of neurofibrils from the end-feet into the nerve cell. The end-feet are, as Held pointed out, the same structures as Deiters observed in macerated preparations fifty years earlier. But while Deiters described them as points from which a secondary system of axons grew out from the cell, Held considered them to be the terminations of fibres *upon* the cell. Structures apparently similar to the end-feet of Held may be observed with ease in Golgi preparations, generally on the cell dendrites. But just as in a previous paper (25) I have argued that the cell dendrite is a protoplasmic outgrowth from the nerve cell which has come to surround a group of neurofibrils that have penetrated into the body of the nerve cell, so it seems to me that the end-feet of Held are to be looked upon as minute dendrites, so to speak, which have come to surround the delicate terminal branch of a collateral as it grew into the substance of the cell (see figures 2 and 4).

SUMMARY.

1. The teaching of Cajal, that there is protoplasmic discontinuity between the elements of a reflex arc has been replaced in recent years by the knowledge that the terminations of motor nerves upon muscle are definitely hypolemmal; whilst the ultimate ramifications of the sensory nerves in the receptive organ are intraprotoplasmic and not interprotoplasmic, as Cajal, Retzius, and others have maintained (Boeke). In previous papers I have described a protoplasmic

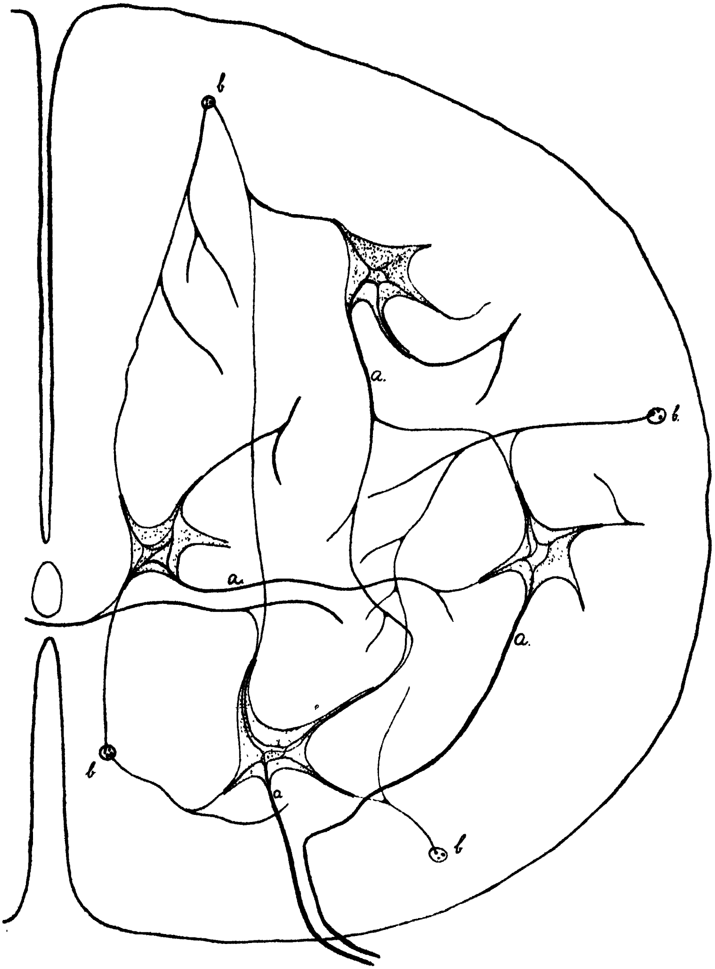

Figure 5. Schematic section of spinal cord, showing relation of collaterals to nerve cells. At **b** are shown four transversely cut, longitudinally running fibres of the white matter, whose neurofibrils are indicated. Collaterals arise by the separation out of neurofibrils (Bethe, Lenhossek). The collaterals branch in the grey matter. Many of the branches are cut short as they leave the plane of the section; others enter the nerve cells, and are recognizable as neurofibrils within the cells. The axons of the nerve cells are represented by *a*; no attempt has been made to represent the neurofibril composition of the axons. The perinuclear anastomosing neurofibril network is indicated in the four nerve cells.

continuity of the neurone junctions within the central nervous system. As the theory of contact synapses has been so generally accepted, the evidence on which this theory is based is here critically reviewed.

2. The theory was formulated by Cajal, Waldeyer, and others upon histological studies of Golgi preparations, and on the indirect evidence obtained from degeneration experiments, and observations on embryonic tissue. It is pointed out that the results obtained from the last two methods apply also to the motor nerve endings on muscle; nevertheless, the motor nerve ending exhibits protoplasmic continuity, and not contiguity.

3. The evidence of Golgi preparations is not decisive; it rests upon the fact that in some preparations axons and their collaterals stain deeply, while the nerve cells do not stain; the collaterals also very frequently end freely. It is pointed out, however, that this free ending of collaterals is valueless as evidence in support of a discontinuity at the neurone junction, since, if we trace the collateral backwards towards the white matter, it again very frequently ends blindly, definitely due to incomplete staining. The Golgi method therefore does not provide a complete picture of the neurone collaterals.

4. Subsequently the evidence for the theory of contact was amplified by Cajal by studies on reduced silver material. In such preparations fine collaterals were described ending in minute "end buttons" close to the body of the nerve cell. Held, on the contrary, found these "end buttons" to be in direct continuity with the cell protoplasm.

5. My own studies lead to the conclusion that the collaterals, which have arisen from the longitudinal axons of the white matter by the separation out of individual neurofibrils (Lenhossek, 19; Bethe, 3), traverse the grey matter of the cord, and after undergoing considerable branching penetrate the dendrites of the nerve cells; they pass as neurofibrils along the dendrites to the middle of the cell, where they form a perinuclear network, which Cajal himself observed, and from this network a relatively small number of neurofibrils enter the axon (see figure 5). The conclusion seems unavoidable that the neurofibril is the conducting unit of the central nervous system. Except for the fact that no anastomosis of fibrils occurs in the intercellular part of the grey matter, my results are identical with those obtained by Apathy on invertebrate tissue.

6. It is pointed out that the evidence from Golgi preparations is not incompatible with this theory. The method has its limitations, because it cannot reveal the internal organization of the nerve cells. The reduced silver method alone is competent to do this, but even with this method Cajal does not seem ever to have observed neurofibrils of the nerve cell emerging from the dendrites and traversing the grey matter as naked fibrils for very great distances.

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THE LIVER GLYCOGEN AFTER PARTIAL PANCREATECTOMY IN THE GUINEA PIG

by

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(Submitted for publication 18th July, 1927.)

In a recent investigation of pancreatic regeneration in the guinea-pig by G. R. Cameron (1) the opportunity was afforded me of investigating the glycogen content of the liver and the residual content of adrenaline in the supra-renals in a large number of guinea-pigs. This experimental material was divided into three main groups, and each of these into two sub-groups. In the animals of Group 1 (a) a portion of the pancreas had been removed, while those of Group 1 (b) were normal controls from the same litters as the operated animals. In Group 2 (a) the guinea-pigs, like those of 1 (a), had a portion of the pancreas removed, but received a daily injection of one unit of insulin subcutaneously for a period of some months. Group 2 (b) was composed of control animals from the same litters as those of 2 (a), which received the same doses of insulin, but were not operated on. In the guinea-pigs of Group 3 (a) part of the pancreas was removed, and they received daily ·05 c.cm. of pituitrin (Commonwealth Serum Laboratory) per kilo of body weight. Group 3 (b) was composed of control animals from the same litters are 3 (a), which received similar pituitary injections, but were not subjected to operation.

The degree of pancreatic deficiency caused by the operations was not severe, as the sugar tolerance curves obtained during the progress of the experiment showed no abnormality, except in animals of Group 3, which were receiving injections of pituitrin.

The average growth curves of all the groups were normal until the last two months of the experiment, when the injected animals lost some weight, and the dosage of insulin and of pituitrin had to be decreased.

The animals were killed in the same order as that in which they had been put up in cages and operated upon 113 to 314 days from the date of operation, and in the injected animals 89 to 278 days after these were commenced. Control animals were killed as far as possible on the same days as their litter mates.

Methods.

The animals were killed almost instantaneously by dividing the vessels in the neck after producing unconsciousness by a blow on the head. The liver and adrenals were removed and weighed, and the whole of these organs were at once used for the estimations. Glycogen was determined by Cole's modification of Pflüger's method (2), adrenaline by the method of Folin, Cannon and Dennis (3), using the slight modifications described by Kellaway (4). Estimations of the urea in the blood at death were also made in many cases by Maclean's modification (5) of Van Slyke's method.

Results.

The results are shown in Tables 1, 2, and 3. In these the alphabetical letters indicate the litter to which the animals belonged; the body weights are the weights of the animals less the blood shed at death and the contents of the alimentary canal and urinary and gall bladders; glycogen is expressed as percentages of fresh liver weight, adrenaline in mgms. per kilo of body weight, and urea in mgms. per 100 cc. of blood.

Glycogen estimations were carried out in 18 operated animals of the first group, and varied between 0·48% and 6·09%, the mean value being 1·75%. In the control group, among 14 observations the extremes were 0·61% and 3·46%, and the mean was 1·81%. There appeared to be no appreciable alteration in the storage of glycogen in the liver following the removal of a large portion of the islet tissue of the pancreas. In view of the large variations in the value for glycogen the observations are probably too few. The means in the two sub-groups are, however, almost identical; a result which was expected, as the animals which had been subjected to operation were in good condition, and had shown no disturbance of carbohydrate metabolism, giving normal sugar tolerance curves during the course of the experiment. In these animals Cameron found quantitative evidence of the regeneration of islet tissues.

In the second group which received insulin in both operated and control animals there was a diminution in the amount of glycogen stored. This was more marked in the unoperated group, in which the mean value of 23 observations was ·90%, as against 1·32% for 18 animals in which a part of the pancreas had been removed.

The administration of insulin to normal rabbits, both in the fasting and in the fed condition, has been shown by Macleod and McCormick (7) and by Dudley and Marrian (8) to cause marked depletion of the glycogen in the liver. The present observations seem to show that while the insulin administered to these animals stimulated glycogenolysis, it was aided in this action by insulin secreted by the islet tissue. Now Cameron's experiments showed that the administration of insulin inhibits the regeneration of islet tissue which occurs after removal of a part of the pancreas, and, in agreement with his findings, my results suggest that the output of insulin from the pancreas is less when a portion of the pancreas has been removed and when regeneration is prevented from occurring.

In the third group in which pituitrin was injected the glycogen values were 1·44% in 20 operated and 1·59% in 23 control animals. Whether these lowered mean values, 1·44% and 1·59% as compared with 1·75% and 1·81% in Group 1 are significant, is open to question. The diminution cannot readily be explained by any direct action on the liver, since pituitrin appears to oppose glycogenolysis caused by the injection of adrenaline Stenström (9), and the antagonism between pituitrin and insulin demonstrated by Burn (10) might have led us to expect that in animals in which pituitrin was injected decreased glycogenolysis would have occurred, and would have been reflected in values for the liver glycogen rather higher in these animals than in those of Group 1. Cameron has, however, shown that the injection of pituitrin causes in both normal and operated animals a marked increase in the proportion of islet tissue. It is probable, therefore, that repeated injections of pituitrin in these animals stimulated the pancreas to increased production of insulin, and after the effect of the last pituitary injection had passed off the excessive output of insulin determined an increased amount of glycogenolysis.

The results obtained for the residual adrenaline in the suprarenals are more difficult to interpret, though generally speaking high glycogen values are found with low amounts of residual adrenaline, and *vice versa*. This is not clearly reflected in the mean values, for in some animals glycogen and in others adrenaline was not estimated.

In Group 1 (a), in which a portion of the pancreas was removed, but in which regeneration was found by Cameron to have occurred, the mean value of 18 observations was ·371 mgms. per kilo. The mean value of 10 observations in the control group was ·287 mgms., a result which is almost certainly too low, and must be disregarded, as the number of estimations on which it is based is too small.

In the group in which insulin was injected the mean value of 18 observations on operated animals, Group 2 (a), was ·334, as compared with ·365, the mean of 22 observations in the control group, 2 (b). The influence of insulin in operated animals in which regeneration of the islet tissue has been prevented from occurring (Cameron) appears to have been to cause a lowered residue of adrenaline in the gland, ·334 as compared with ·371 mgms. per kilo in the operated group without insulin (Group 1 (a)). This result, if significant, is very difficult to explain. Cameron, McIvor and Bliss (11) and Houssay, Lewis and Molinelli (12) have shown that the output of adrenaline is increased during insulin hypoglycaemia. As the last injection of insulin was given in these animals 24 hours before death, it is unlikely that this dose, even if it caused hypoglycaemia, would affect the residual store of adrenaline, and we are therefore concerned only with the insulin normally produced by functioning islet tissue in the two sets of animals. The general inverse relationship between the amount of glycogen in the liver and the adrenaline stored in the suprarenal would have led us to expect a lower residual adrenaline in the operated group, where the mean value of glycogen is higher. Since as long as glycogen is present in the liver adrenaline can cause glycogenolysis and compensate for the lowering of blood sugar caused by insulin, it is conceivable that in these animals the low residual content of adrenaline may be attributable to diminished need for storage when a normal amount of insulin can no longer be secreted.

Finally, in Group 3, in both operated and unoperated animals in which pituitrin has been injected, and in which, as Cameron showed, over-production of islet tissue occurred, the values for residual adrenaline were ·384 and ·387 mgms. per kilo, a finding which is in accord with the hypothesis suggested above.

The estimation of blood urea yielded no significant findings in any of the experimental groups, though it served to indicate the poor condition of animal E.E.2, which was moribund when killed, and in which it reached 177 mgms. In a few other cases in which the animals were apparently in good condition, the blood urea was higher than normal.

CONCLUSIONS.

1. Prolonged daily injections of small doses of pituitrin to guinea-pigs leads to diminution of the glycogen content of the liver. A similar but more marked change, which is to some extent compensated if a portion of the pancreas has been removed, follows the daily injection of insulin. Partial pancreatectomy by itself is without effect.
2. The residual adrenaline in the suprarenals appears to be diminished following the injection of insulin in guinea-pigs in which a portion of the pancreas has been removed, though not in normal animals. Somewhat higher values were obtained following the injections of pituitrin.
3. No significant changes in blood urea were observed in these experiments.

ACKNOWLEDGMENTS.

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TABLE 1.

Partial Pancreatectomy Alone.

Operated: Group 1 (a).						Controls: Group 1 (b).					
Animal.	Sex.	Weight.	Glycogen.	Adrenaline.	Urea.	Animal.	Sex.	Weight.	Glycogen.	Adrenaline.	Urea.
A1	M	626	0·79	0·279	49	A2	F	667	—	—	—
H1	M	637	0·95	0·342	45	H4	M	641	1·43	0·217	44
H2	M	708	6·09	0·213	43						
X1	M	571	1·01	0·567	63	X2	F	682	1·87	—	—
FF1	M	668	0·49	0·408	65	FF2	M	695	1·20	0·355	55
GG1	M	562	1·18	0·539	45						
OO1	M	533	0·58	0·413	—	OO2	M	660	0·61	0·333	55
PP1	M	535	0·48	0·359	63	PP2	M	688	2·04	—	—
LLL1	M	643	2·08	0·472	48	LLL4	M	830	3·29	0·317	41
D1	F	585	4·33	0·291	54	D2	M	669	1·80	0·267	42
K1	F	742	3·32	0·246	54	K2	F	650	2·41	—	—
S1	F	718	1·70	0·313	45						
V1	F	658	0·60	0·375	48						
V2	F	611	1·11	0·376	48	V3	F	640	1·28	0·301	65
Y1	F	549	0·82	0·453	47						
UU1	F	632	1·49	0·339	22	UU3	F	770	1·07	0·277	58
UU2	F	610	2·98	0·346	30	UU4	F	682	1·14	0·322	—
BBB1	F	624	1·49	0·354	56	BBB2	M	660	2·46	0·172	99
						PP3	M	667	1·33	0·310	37
						O2	F	673	3·46	—	—
Mean	M		1·52	0·399	53	Mean	M		1·77	0·281	55
,,	F		1·98	0·344	45	,,	F		1·87	0·300	62
Total			1·75	0·371	49	Total			1·81	0·287	56

TABLE 2.

Pancreatectomy and Insulin.

Operated: Group 2 (a).						Controls: Group 2 (b).					
Animal.	Sex.	Weight.	Glycogen.	Adrenaline.	Urea.	Animal.	Sex.	Weight.	Glycogen.	Adrenaline.	Urea.
B1	M	762	3·62	0·209	40	B3	M	626	0·65	0·309	66
E1	M	599	0·72	0·308	57	E2	F	579	0·80	0·323	49
L1	M	614	1·40	0·236	95	L2	F	585	0·39	0·542	53
HH1	M	654	3·31	0·379	43	HH2	M	633	2·08	0·458	—
MM1	M	638	0·43	0·291	50	MM2	F	691	1·59	0·267	51
RR1	M	704	1·31	0·360	65	RR2	M	612	0·63	0·398	30

Animal.	Sex.	Weight.	Glycogen.	Adrenaline.	Urea.	Animal.	Sex.	Weight.	Glycogen.	Adrenaline.	Urea.
SS1	M	641	1·33	0·312	37	SS2	M	700	1·26	0·304	55
SSS1	M	667	2·09	0·290	50	SSS2	F	600	0·96	0·383	42
B2	F	682	1·72	0·329	41						
P1	F	553	0·32	0·298	70	P2	M	812	0·22	0·367	46
CC1	F	530	0·99	0·630	55	CC2	F	800	2·13	0·371	63
DD1	F	610	0·58	0·401	52	DD2	F	644	0·59	0·150	64
VV1	F	594	2·08	0·311	—	VV2	F	642	0·85	0·370	37
WW1	F	585	0·64	0·441	55	WW2	F	547	0·94	0·463	40
AAA1	F	636	0·46	0·337	45	AAA2	M	671	0·65	0·345	40
CCC2	F	647	0·49	0·257	52	CCC3	F	529	0·79	0·391	67
DDD1	F	822	0·66	0·243	52	DDD2	M	670	0·79	0·338	—
MMM1	F	608	1·61	0·424	48	MMM4	M	590	0·82	0·415	47
						HH2	M	659	0·81	—	—
						AA3	M	680	—	0·360	36
						Z2	F	576	1·04	0·408	56
						AA2	F	680	0·46	0·316	55
						HH3	F	600	0·99	0·436	48
						DDD3	F	635	0·80	—	56
						AAA3	F	720	0·40	0·320	47
Mean	M		1·78	0·298	55	Mean	M		0·89	0·366	46
„	F		0·96	0·364	52	„	F		0·90	0·365	52
Total			1·32	0·334	53	Total			0·90	0·365	49

TABLE 3.

Pancreactectomy and Pituitrin.

Operation: Group 3 (a).						Controls: Group 3 (b).					
Animal.	Sex.	Weight.	Glycogen.	Adrenaline.	Urea.	Animal.	Sex.	Weight.	Glycogen.	Adrenaline.	Urea.
C1	M	713	1·26	0·314	41	C3	F	428	1·18	—	—
N1	M	500	1·60	0·484	65	N3	M	709	3·03	0·352	56
Q1	M	585	3·75	0·319	—	Q2	M	708	0·37	0·296	55
T1	M	801	0·44	0·337	50						
H1	M	695	3·69	0·343	58	H2	M	691	2·68	0·340	55
LL1	M	685	0·78	0·363	57	LL2	F	600	1·21	0·423	54
QQ1	M	559	1·10	0·429	62	QQ2	M	570	1·43	0·444	57
KKK1	M	608	1·11	0·404	67	KKK2	M	652	1·42	—	—
LLL2	M	695	1·55	0·328	66	LLL3	F	663	1·53	0·422	30
C2	F	633	0·86	0·350	47						
F1	F	636	0·69	0·388	47	F2	F	666	3·26	0·388	34

Animal.	Sex.	Weight.	Glycogen.	Adrenaline.	Urea.	Animal.	Sex.	Weight.	Glycogen.	Adrenaline.	Urea.
S1	F	661	2·44	0·363	56	S2	M	717	1·89	0·374	43
M1	F	730	0·65	0·315	50						
N2	F	495	—	—	—	N4	F	560	3·33	0·426	63
Y2	F	653	1·68	—	—	Y3	F	631	0·60	0·468	49
BB1	F	453	—	—	—	BB2	F	720	0·43	0·377	60
EE1	F	569	0·71	0·522	60	EE2	F	524	0·50	0·103	177
XX1	F	534	0·55	0·491	57	XX2	F	550	0·66	0·434	71
YY1	F	650	2·30	—	—	YY2	F	641	0·69	0·369	61
EEE1	F	785	1·41	0·303	35	EEE2	F	662	0·88	0·374	44
FFF1	F	671	1·15	0·399	38	FFF2	F	610	1·27	0·371	56
MMM2	F	579	1·11	0·464	56	MMM3	F	560	1·49	0·408	47
						YY3	M	680	1·57	0·387	44
						KKK3	M	681	3·48	0·440	49
						HHH1	M	649	3·14	0·468	66
						G1	F	672	—	0·513	63
						TT2	F	611	—	0·364	—
						EE3	F	629	0·71	0·349	71
Mean	M		1·70	0·369	58	Mean	M		2·11	0·388	53
„	F		1·23	0·399	49	„	F		1·27	0·387	63
Total			1·44	0·384	54	Total			1·59	0·387	60

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A SIMPLE SPECTRO-COLORIMETER

by

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The instrument to be described was constructed for the rapid optical estimation of substances whose solutions show well-defined absorption bands in the visible region of the spectrum, in the presence of other substances having a less selective absorption in this region. Such estimations are usually carried out on a spectro-photometer, but its cost and complexity place it out of the reach of many smaller laboratories and institutions. During the initial experiments on globin, carried out by Hill and Holden (1), an approximate estimation of the concentration of globin in the solutions was made by adding to such a solution an excess of haematin in dilute sodium carbonate, reducing the resulting methaemoglobin to haemoglobin, and oxidizing the latter to oxy-haemoglobin with atmospheric oxygen. A solution of oxy-haemoglobin of known concentration was then diluted with known volumes of water until the intensity of its α absorption band appeared to equal that of the α band of the resynthesized oxy-haemoglobin. The comparison was made in two similar test-tubes, using a small hand spectroscope. Mr. Hill suggested that a colorimeter might be substituted for the two test-tubes, and a few preliminary trials were made. When the author left Cambridge it was decided that he should construct a suitable apparatus and endeavour to determine its applicability.

The metal stand of the apparatus was kept as simple as possible, in order that the cost might be as low as was consistent with efficiency. It consists of a cast-iron base, into which are screwed two upright iron rods, which carry simple adjustable screw clamps for holding the spectroscope, lens, and prism. The colorimeter is fastened to the base with clips. The optical parts comprise a Bausch and Lomb 40 mm. biological colorimeter with solid prisms, a 50 mm. microscope objective screwed into a short length of brass tube, a direct-vision spectroscope, and a 25 mm. right-angle prism with its reflecting face silvered. The source of illumination is a 40 w. gas-filled electric lamp with an opal globe, placed about 25 cm. from the colorimeter mirror in a large lamp-house, fitting closely to the front of the colorimeter, in order to exclude stray light. The eye-piece of the colorimeter is removed, and the image of the line between the two fields of the instrument focussed by the objective on the slit of the spectroscope. The right-angle prism is placed between the objective and the spectroscope to enable the latter to be in a horizontal position, the person using the instrument to remain seated. If the slit be vertical, two spectra are seen, corresponding to the two fields of the colorimeter. In using the instrument the "standard" cup is so adjusted that the chief band in the corresponding spectrum is of a convenient intensity. The "unknown" cup is then moved until the same band on that side equals in intensity that on the "standard side." The calculations are the same as for an ordinary colorimeter. In the photograph the lamp-house has been removed, in order to show the other portions more clearly.

Three examples may indicate some of the uses of the spectro-colorimeter.

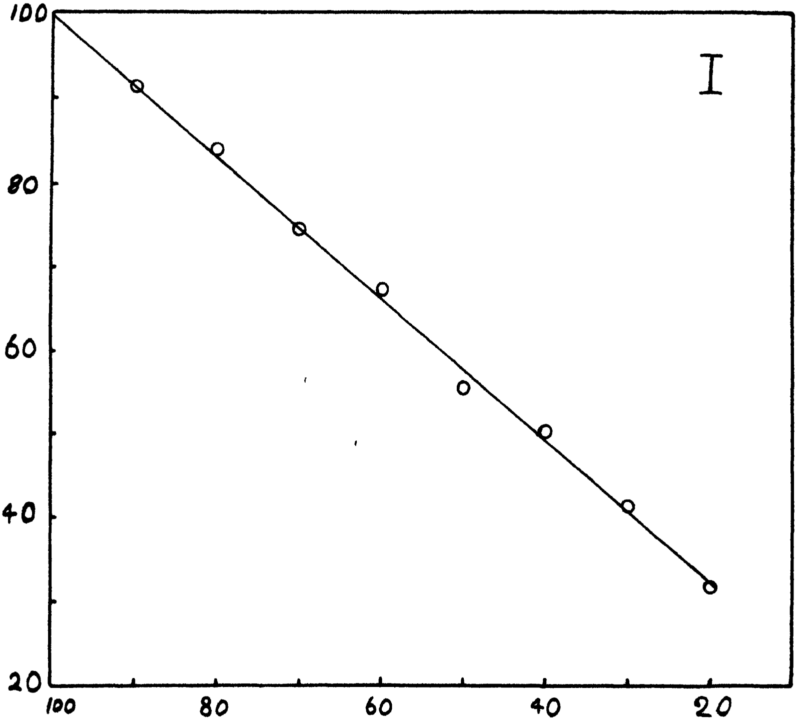
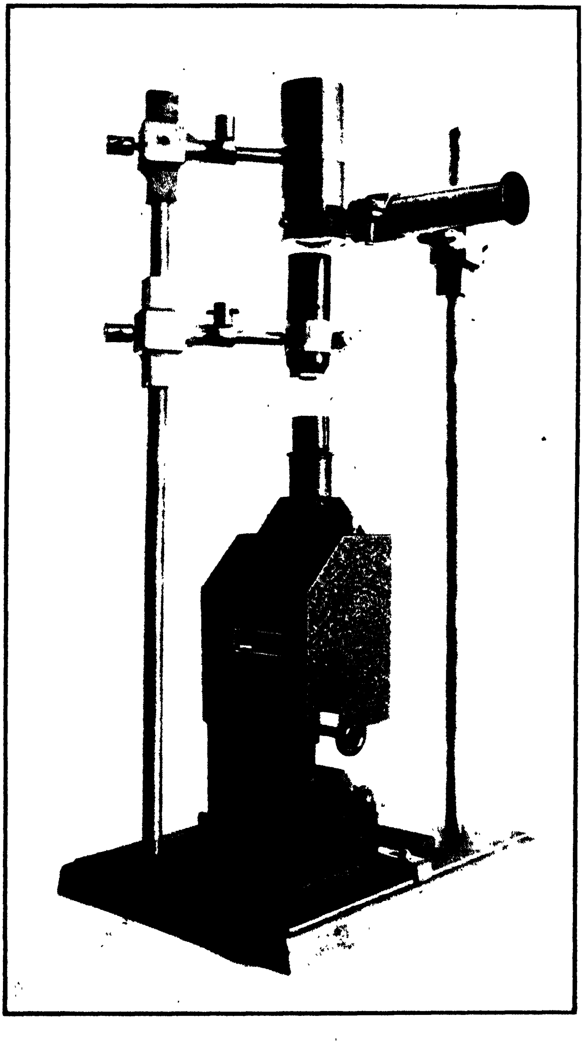


Figure 1.

Ordinates. Apparent amount of oxy-haemoglobin present.
Abscissae. Percentage of pigment present as oxy-haemoglobin, the remainder being alkaline haematin.

From a dilute solution of oxy-haemoglobin a series of other solutions of known relative concentration were prepared by dilution with water. The concentration of oxy-haemoglobin in each was then estimated relatively to that of the stock solution by comparison in the spectro-colorimeter. The results obtained follow:



Actual	..	100	83·3	71·4	62·5	55·6	50·0
Estimated	..	100	83·3	71·9	62·9	56·2	50·2

In such operations as the gradual denaturation of oxy-haemoglobin, where the total molecular concentration of the pigment is known, the proportion which is combined with globin may be determined by applying the correction for the effect of the free haematin present derived from the curve in figure 1. This curve was obtained by estimating the apparent concentration of oxy-haemoglobin in a series of known mixtures with alkaline haematin, so chosen that the total molecular concentration of pigment remained constant. An arbitrary standard of pure oxy-haemoglobin was used.

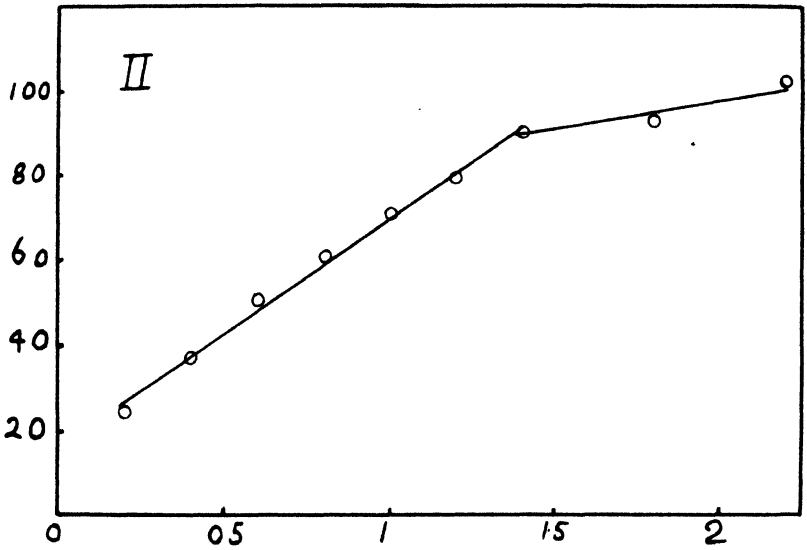


Figure 2.

Ordinates. Oxy-haemoglobin, estimated in arbitrary units.
Abscissae. c.c. of alkaline haematin added. 1 c.c.–0·356 mgm. of haemin.

The instrument may also be used instead of the spectro-photometer for the estimation of the concentration of globin in a solution by titration with haematin (1). Known weights of haematin in sodium carbonate solution are added to suitable measured samples of the globin solution. The pigment is reduced with Stokes' reagent and oxidized by gentle aeration. The amount of oxy-haemoglobin so obtained is estimated against a fixed standard. The discontinuity in the curve in figure 2 marks the point where sufficient haematin has been added to combine with all the globin.

In a few cases the spectro-colorimeter has been employed for the comparison of the intensities of the absorption bands of the spectra of haemochromogens. Many of these compounds, however, form insoluble aggregates, the suspensions of which are unsuitable for spectro-photometric work.

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REFERENCE.

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THE BASAL METABOLIC RATE OF STUDENTS IN SYDNEY, N.S.W., WITH A DISCUSSION ON METHODS OF DETERMINING BASAL METABOLISM

by

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During the past ten years a considerable amount of data has been collected and published concerning the so-called basal metabolic rate of the body. The greater part of this work has been carried out in America, and the normal standards used for comparison have been worked out for American people. It was thought that it would be interesting to see how the standards would apply in Sydney, New South Wales. The basal metabolic rate has been determined on the students attending the third year course in Medicine at the University of Sydney, and from the results obtained it was found that, using the Du Bois standard for comparison, the average basal metabolic rate was 8·9% lower in men and 10·5% lower in women. Using the Harris and Benedict standard for comparison, the average basal metabolic rate was lowered by 7·6% in the men and 8·6% in the women.

Method.

The method used for the determination of the basal metabolic rate was the open circuit or gasometer method of indirect calorimetry. With this method the subject inspires atmospheric air, and the air expired in a definite time is collected in a Douglas bag. The volume expired is measured, and a sample is removed for the determination of its oxygen and carbon dioxide content. The percentage of oxygen absorbed is then calculated, and the respiratory quotient from which the heat production for 24 hours can be determined.

Preparation of the subject.

By the basal metabolic rate is understood that level of metabolism of the body when the individual is in a post-absorptive condition, in complete muscular repose, and after all exciting influences have been removed. The post-absorptive condition is considered to be reached 12 hours after food, and complete muscular repose is obtained by a preliminary rest of half an hour.

The experiments were carried out during the Lent and Trinity terms of 1926, that is, during April, May, June, July, and August; mostly at 11 a.m. and

12 noon; a few were earlier, namely, at 9 a.m. and 10 a.m. Since the determinations were carried out during the autumn and winter weather, it was considered advisable for students whose basal metabolic rates were not being determined until 11 a.m. or 12 noon to be allowed a light breakfast, rather than ask them to travel anything up to one and a half hour's journey to the University without taking any food at all. Subjects were therefore allowed a cup of tea and two slices of toast for breakfast, which would be taken about 7.30 or 8 a.m. Benedict (6) has shown that a light breakfast of 250 calories, provided it contains little or no protein, increases metabolism by about 2% two hours after ingestion. Soderstrom, Barr and Du Bois (34) allowed their subjects a breakfast consisting of a slice of bread, 8 grms. of butter, one cup of caffein-free coffee, 10 grms. of sucrose, and 66 cc. of milk = 222 calories. This gave an average increase of 7% in the first hour and 2% in the second and third hours in the metabolism of the body.

The question whether the true basal metabolic rate can be obtained on subjects at 11 a.m. and 12 noon, after the muscular movements and activity incidental to the usual routine of a morning's work needs consideration. Benedict and Crofts (7) found practically no increase in basal metabolic rate when measured after rising, bathing, dressing, walking in winter weather for ten minutes, climbing three flights of stairs, and then resting for thirty minutes in a warm room, over that measured before rising, with the body well covered, warm, and relaxed. This finding, they claim, justifies making basal metabolic rate experiments after one-half hour's rest, when it is necessary for the patient to leave home and travel to the laboratory or hospital. Barach and Draper (2) also studied this question, and they published results on a series of patients in hospital and on out-patients who had walked to the hospital, and had thus been exposed to changes of weather and clothing. Provided basal conditions were rigidly observed, no variation in the basal metabolic rate could be detected as a result of the movement, etc., required to reach hospital between these patients and those who had slept at the hospital and had not risen.

The basal metabolic rate of subjects 50 and X, determined at 6.30 and 7 a.m., before rising, and those determined at later hours in the day, at 10 a.m. or 11 a.m., after travelling to the University, show no appreciable difference.

Subject X.

Time.	B.M.R.
6.40 a.m.	1,057 cals.
6.30 a.m.	1,031 cals.
10 a.m. to 12	1,046 cals. (average)

Subject 50.

Time.	B.M.R.
7 a.m.	1,411 cals.
10 a.m. to 12	1,408 cals. (average)

From these figures we can take it that the basal metabolic rate of subjects determined at 11 a.m. and 12 noon was not appreciably increased by the usual routine of the morning's work since they rested quietly for one half-hour before the sample of expired air was collected.

Apart from food and muscular movement, the most important factors which can affect the basal metabolic rate are a rise in body temperature and nervous stimulation. It is well known that fever is attended with an increased metabolic rate, and Du Bois showed that this increase in febrile diseases follows van Hoff's law of velocity of chemical reactions with change of temperature. Du Bois showed that since the velocity of chemical reactions increases between two to three times with a rise of 10°C., then a rise in body temperature from 37°C. to 40°C. increases the basal metabolic rate from 30% to 40%, or for every 1°F. rise of body temperature above normal an increase in basal metabolic rate of 7·2% takes place. The mouth temperatures of a large number of the subjects of this investigation were recorded, and there was one record of a temperature above 99°F.

The degree of nervous excitement is, of course, much more difficult to estimate, or even guess at. The room in which the experiments were carried out contained two beds, on which the subjects rested, lying quietly for the necessary half-hour. A fire, to keep the room comfortably warm, was lighted on the cooler days. One realizes that it is a question how much lying down, apparently still, can be taken as a criterion of complete rest and muscular relaxation, and this factor applies equally to patients in hospital as to subjects of this investigation.

The pulse rate of a number of subjects was recorded after the half-hour's rest, and in some cases again ten minutes after commencing the expiration into the Douglas bag. It was thought that the pulse rate would be an indication of the state of rest, that if a subject was nervous or worried by the procedure the pulse rate would probably be increased during the collection of the sample of air. No clear correlation could be established from the results obtained between pulse rates and the basal metabolic rates.

Benedict (3) in 1914 claimed that there is a clearly established relationship between the pulse rate and total metabolism of an individual, and that a reduction, under basal conditions, of 10 beats per minute in pulse rate is associated with an appreciable reduction in basal metabolic rate.

In Tables 3 and 5 a number of basal metabolic rates and pulse rates on one subject are recorded, and it can be seen that a lowering of the pulse rate by 10 beats per minute does not necessarily coincide with a reduction in the basal metabolic rate. These results agree with those of Blunt and Dye (8), who found no relation between minimum pulse rate and basal metabolic rate, whether on the same individual or on a number of individuals.

The days of minimum pulse rate do not always coincide with the days of minimum basal metabolic rate, and certainly persons with a low pulse rate do not have the lowest basal metabolic rates as compared with the figures for their standard metabolism. However, in the later experiments a record of pulse rate was made after the half-hour's rest, just before the air sample was collected, and again during the collection of the sample, ten to twelve minutes after commencing breathing into the bag. If these two pulse rates do not agree it suggests that the subject is worried by the procedure, and the unusual condition of breathing through the mouth for some time; this indicates that a true basal sample is not being obtained. The results obtained by recording these two pulse rates did not, however, give any information, since in only two cases did the second pulse rate differ from the first by more than 5 beats per minute, and the basal metabolic rate in these two cases agreed well with the determinations made when the pulse rate did not alter appreciably. Still, when determining the basal metabolic rate of patients the record of these two pulse rates may serve to give information about the nervous condition of the patient.

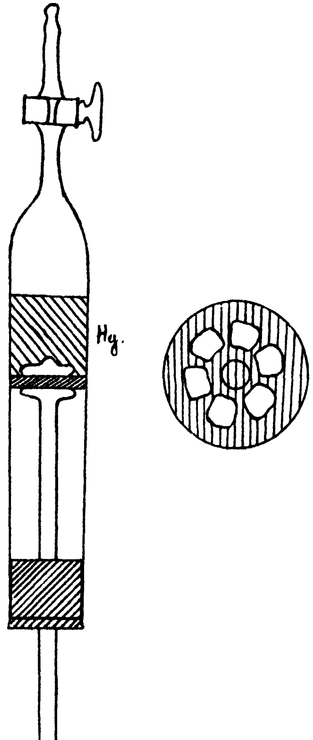
The basal metabolic rate was determined at least twice on each subject, and if the results did not agree within 10% of each other, a third determination was carried out. The determinations were carried out a week apart, the subjects being given a definite time on a certain day of the week, and kept that time for three weeks.

In some cases it was noted that the first determination was higher than the following determination. This has been considered as probably due to a slight nervousness and the unnatural procedure of breathing through a mouthpiece. These factors tend to cause abnormal respiration with irregular rhythm. Associated wtih this there is usually a quickened respiration rate and a higher volume output of air. When the basal metabolic rate is determined a second and third time these factors are greatly decreased, the subject becomes used to the procedure and the apparatus, and breathes more normally and relaxes more completely. Subjects themselves have remarked after the second and third experiment that they felt more at ease that day while giving the sample, and that they expected a truer basal figure would be obtained.

Apparatus consists of a nose-clip, mouthpiece, Douglas bag, and tubing. To the metal box of the mouthpiece were attached two pieces of non-collapsible rubber tubing. Near the junction of each tubing with the mouthpiece was placed a valve, one being an inlet the other an outlet valve. The valves were made of thin sheet rubber, which fitted on flat to a nickelled disc, which had been perforated as in the diagram. The two valves were exactly the same, but reversed in direction. The valves were tested by placing a lighted match at the free end of the tube and breathing through the mouthpiece; if no movement of the flame was obtained at the end of the tube attached to the inlet valve when an expiration was made the valve was considered airtight. Similarly the outlet valve was only considered true if no movement of the flame at the free end of its tubing was obtained during inspiration. If the valves did not stand this test they were discarded.

The tubing attached to the inlet valve was left open to the air, so that the subject breathed atmospheric air. The free end of the other tubing was attached to a Douglas bag of 130 litres capacity. A tap was attached to the tubing near the bag, so that the expired air could be directed into the room, or by turning a tap, into the Douglas bag.

The subject, after adjusting the mouthpiece, was allowed to breathe through it for five minutes before the expired air was directed into the bag; this was done to give the subject time to get used to the mouthpiece and for the respiration to become regular.



For convenience in calculation the air expired was collected for a period of 14·4 minutes, since this is exactly $^1/_{100}$ of 24 hours, the timing being done by the operator by means of a stop watch.

As soon as the sample was collected the bag was well shaken to mix its contents thoroughly, and the air was passed through a dry meter. A thermometer was placed in the meter to record the temperature of the air, and the barometric pressure was recorded at the same time. When about half the contents had been expelled from the bag, a sample of the expired air was collected by means of a side tube, placed in the tubing between the tap and the bag. The sampling tube was of the nature of a syringe, consisting of about 6 inches of 1-inch glass tubing, provided at one end with a glass tap, and fitted with a glass plunger with a rubber disc. About 1 inch of mercury was placed in the tube above the plunger to act as a seal; the whole was well lubricated with glycerine (see diagram).

When the tube was filled with the air sample the tap was also sealed with mercury. These sampling tubes which held 66 cc. to 80 cc. of air, were quite air-tight, and by simply raising the plunger, so that the tap and its tube were filled with mercury, connecting the tap to the side tube of the bag, and then drawing down the plunger, a sample of the expired air was easily obtained. After connection to the bag the plunger was raised and lowered several times, drawing the air from and expelling the air into the bag and tubing, so a very well mixed sample of the expired was obtained. This type of sampling tube was found to be much simpler to use than the old Hempel tube, with its necessary mercury reservoir. The sampling tube was designed by Sir C. J. Martin, Director of the Lister Institute, London.

The meter used was an ordinary dry meter, and by being placed in series with a standard wet meter, was tested (1) by driving air through the two meters by means of a Crowel blower, and (2) by filling a Douglas bag with air, and then connecting the bag to the meters and expelling the air from the bag by pressure at the rate that is used in measuring the volume of air expired after an experiment. The maximum error of the meter was found to be 2%; the average error was less than 1%.

The analysis of the expired air was carried out by means of a large Haldane apparatus. The apparatus was calibrated by numerous analyses of atmospheric air, and every few weeks check air analyses were performed. The error allowable in the analyses for duplicate figures was CO_2 0·04%, and O_2 0·06%.

As far as possible the air samples were analyzed on the day they were collected, but sometimes they were allowed to stand till the next day, and in a few cases the samples were not analyzed for two days after collection. To test the sampling tubes some air samples were collected in the usual way, and the air analyzed the day the sample was collected, and after standing one, two, and three days. No appreciable alteration in the composition of the air could be detected.

Date.	CO_2	O_2
6–7–26	3·30%	18·11%
7–7–26	3·30%	18·08%
23–6–26	3·50%	16·59%
25–6–26	3·49%	16·61%
25–6–26	3·85%	16·12%
28–6–26	3·83%	16·13%

Three sampling tubes were filled from the same bag at varying stages of passing the contents through the meter. The composition of the air in the three tubes was:

CO_2	3·72%	3·72%	3·71%
O_2	16·86%	16·89%	16·90%

From the above figures it is clear that a good average sample is obtained of the expired air from the Douglas bag, and that no alteration in the composition of the sample collected takes place by leaving it to stand overnight in the sampling tube for analysis.

The determination of the basal metabolic rate was made on 76 subjects, 26 women and 50 men. The subjects were first measured and weighed, and their ages recorded. The standard basal metabolic rates were then calculated according to the Du Bois chart and table, and from the formula of Harris and Benedict.

As can be seen from Table 2, the men showed an average decrease in basal metabolic rate, when compared with the Du Bois predicted value, of 8·9%, with a range of variation of —29·4% to +15·0%. When compared with the Harris and Benedict predicted value the average decrease in basal metabolic rate was 7·6%, with a range of variation of —28·9% to +14·2%. The average respiratory quotient of the men was 0·85.

From Table 1 it can be seen that the basal metabolic rate of the women showed on the average a reduction of 10·5% from the Du Bois predicted value, with a range varying from —33·8% to +11·7%. When compared with the Harris and Benedict predicted value the average decrease in the basal metabolic rate was 8·6%, with a range vary from —31·3% to +10·2%. The average respiratory quotient for the women was 0·83.

Boothy and Sandiford (10) have shown that, by the substitution of certain constants in the Harris and Benedict formulae, surface areas of subjects can be calculated, agreeing remarkably with those calculated by the Du Bois formula. The major discrepancies between the basal metabolic rates as predicted by the Du Bois formulae, and the Harris and Benedict formulae are due to differences in value attributed to factors for age and sex. They conclude that the Du Bois formula for the determination of the surface area and the Du Bois normal standards of heat production for each square metre of body surface for age and sex offer the best means at present available for predicting the normal heat production. The average difference between the two standards in this investigation was 30 calories, while the greatest was 111 calories. In 80% of the subjects the Harris and Benedict value was lower than the Du Bois value.

Comparison of Mean Results with Standards.

The fact that the basal metabolic rates obtained in Sydney were almost all lower than the standard values is interesting, especially since 66% of the results were outside the range of ±10%, which is usually given as the range for normal individuals, and 23% were outside the range of ±15%, which Barach and Draper (2) and others consider should be allowed as normal variations.

The subjects examined were presumably all normal individuals of good health. All had been in attendance at lectures at the University for over two years, and no abnormal conditions had been observed. One can take it that the results obtained are average normal values for the class of the community represented by the University students, and between the ages of 19 and 25 years.

Twelve of the subjects were in active athletic training; the average variation of their basal metabolic rates was —5·8% from the Du Bois standard, with a range from +15% to —19·9%.

All subjects, except No. 27, were born in Australia.

The three subjects, X, 49, and 50, on whom a number of determinations were made, show much lower average variations from the standards, namely, —19·5%, —22·4%, and —18·0%, from the Du Bois standards, with an average of —20%. These subjects had had great experience in experiments on respiration, and consequently their figures are probably more nearly basal. (See Tables 3, 4, and 5.)

The so-called basal metabolic rate is the measurement under standard conditions of the activity of the vital organs, the tone of the muscles, and is more or less influenced by the emotional state of the subject. Thus a true basal figure cannot be obtained without the full co-operation of the subject, or if the subject is nervous or worried by the procedure. A person repeatedly tested as a laboratory subject will show a lower basal metabolic rate than the same subject would have had in the rôle of a patient.

Unless the basal metabolic rate determinations of a subject agreed within 10% of each other a third determination was made, but it was seen later that a greater variation than this can be allowed. Benedict (4) found a daily variation in a number of individuals to be on an average 13·9%, with a maximum variation of 31·3% and a minimum of 3·5%. Blunt and Dye (8) found a daily variation of their subjects equal to 13·2%, with a maximum of 28·8% and a minimum of 7·4%.

The basal metabolic rate determinations over a period of three years showed a variation of 23·7% for subject X, with 21 determinations, and for subject 50 a variation of 11·2% was obtained with 12 determinations. (See Tables 4 and 5.)

The cause of the day to day variations is not clear, for nothing the subject does or fails to do, except sleep, can lower the heat production, whereas any slight disturbing conditions, such as restlessness, discomfort, etc., may cause an appreciable increase. Temporary elevations in heat production may be due to emotional stimulation of the adrenals, resulting in a discharge into the circulation of an excess of adrenalin, as has been shown by Boothy and Sandiford

(11). The injection of adrenalin is the quickest method known of increasing the metabolism of the body. Thyroxin also increases the metabolism of the body, but it is much slower in action, and the effect lasts for a much longer time.

The Cause of the Lower Basal Metabolic Rate.

The predicted values for the basal metabolic rates of the body have chiefly been worked out in U.S., America, by Du Bois and Harris and Benedict (23), and in England by Dreyer (15), that is, on people accustomed to cool climates. Dreyer's standard metabolic rate figures are on the whole intermediate between those of Du Bois and those of Harris and Benedict.

In Sydney the warmer climate suggests that the lower basal metabolic rate that has been found in this investigation may be due to a climatic effect. The regulation of body temperature within its narrow limits is obtained by a nicely balanced adjustment between heat loss and heat production. Heat loss takes place by purely physical means of conduction, radiation, and evaporation from the surface of the body. Increase or diminution of heat loss is then regulated by the skin circulation and the activity of the sweat glands. Heat production may, however, be altered in two ways: a chemical heat regulation where there is an increase or decrease in the metabolic activity of the tissue cells, such as must take place in fevers or in hyperthyroidism and hypothyroidism; and secondly, a physical heat regulation, where increase or decrease in heat production is brought about by muscular activity.

There has been much controversy on the effect of environmental temperature on the basal metabolic rate, and the greatest amount of attention in the past has been paid to the consideration of the adjustment to a lowered external temperature. The earlier physiologists, e.g., Rubner and Voit, believed in the so-called chemical regulation of body warmth, while more recently the physical mechanism of the regulation of body temperature has been more generally accepted. It is held that the increase in metabolism at low temperatures is due entirely to the muscular activity caused by shivering, goose-skin, or movement, and that, of itself, external cold has no immediate influence on the metabolism of the resting cell. Eykman (18) in 1897, and Ranke (31) in 1900, both found that the energy exchange of the body is the same in summer as in winter if the same amount of work is carried out.

Morgulis (28), who studied the CO_2 production and oxygen absorption of dogs exposed to various temperatures, obtaining at the same time a graphic record of the behaviour of the animals, came to the conclusion that there is no special mechanism of chemical regulation of metabolism; that the metabolic changes produced under lowered temperatures are either the result of shivering and restlessness or lack of relaxation on the part of the subjects. They are due to differences in muscular tonicity, produced reflexly through cutaneous stimulation. He concludes that the basal metabolic rate may remain unchanged within a temperature range of the environment of 10°C., provided that complete muscular relaxation can be secured.

Rubner (32) found no variation in the metabolism of a dog with alterations in temperature of 20°C. to 30°C., so physical regulation alone sufficed to maintain the temperature of the body at a constant level. At 35°C. there was a decided increase in the heat production, due to a hyperthermia of the cells through insufficient heat loss. Rubner concluded that below 20°C. and above 30°C. a rise in metabolism is obtained, due to chemical regulation, and a minimal metabolism of the body is obtained only when the fasting organism is surrounded by an atmosphere of 30–35°C.

Eykman (19) determined the energy exchanges at rest of Europeans in the tropics and in Europe, and obtained similar figures. His average figures were:

Europeans in Europe	250 cc. of oxygen per minute.
Europeans in East Indies ..	245·7 cc. of oxygen per minute.
Malays in East Indies	251·5 cc. of oxygen per minute.

A study of the food intake of Europeans in Java by Eykman gave an average of 2,400–2,500 calories per day, which is very close to the figure (2,445) obtained by Rubner for inhabitants of Europe.

Other workers, like Glogner (22), Schilling and Jaffe (33), obtained the same results for food consumption of Europeans in the tropics and in Europe, so that it seems that the food intake in the tropics does not differ appreciably from European standards.

From his experiments Eykman (20) came to the conclusion that the metabolism of the body is the same at high as at medium temperatures, and that no adaptation of the organism, subjected to high temperatures, is brought about by a diminution of the energy output of the body.

However, the basal metabolism at high external temperatures is still a matter of discussion. The question whether the body can respond to increases in external temperatures by a reduction in the quantity of heat produced is still undecided. As various workers have found the basal metabolic rates of individuals to be the same in summer as in winter, and also since Eykman (18) found no decrease in the oxygen consumption of white men in the tropics, it has, until very recently, been generally accepted that, under conditions of high external temperatures, the body temperature is regulated purely by physical means.

Breinl and Young (13), from their experiments in Townsville, found in 1920 that the maintenance of body temperature in the tropics is not brought about by any decrease in the heat production of the body. Almeida (1), however, in 1919 determined the basal metabolic rate of 10 white men who were born and living in tropical Brazil. The result of 26 experiments showed an average basal metabolic rate of 30·35 calories per square metre per hour, with a range from 25·67 to 37·56 calories. Almeida concludes that the inhabitants of warm climates have basal metabolic rates very much lower than those of inhabitants of cold or temperate climates. He points out that this is of great importance from the point of view of acclimatization to warm climates, for it represents the acquisition of a new property, that of reducing the production of heat to a very low level not attained by organisms accustomed to low temperatures.

The results obtained by Rubner, Eykman (32, 19), etc., are attributed to the fact that their subjects had not had time to adapt themselves to the new conditions, which seem to take a long time to become established.

Corlette (14), from theoretical observations, prophesied that the inhabitants of Sydney will be found to have a basal metabolic rate 200 to 500 calories lower than the standard value of Du Bois, which represents the heat loss of an average individual in New York. These results Corlette finds confirmed in the figures for total metabolism in the dietary studies of students in Sydney by Wardlaw (35), who found an average food consumption per day of 1,300 calories for men and 1,283 calories for women. These figures are about 500 calories lower than the Atwater standards for U.S. America.

The basal metabolic rate was found in this investigation to be on an average 150 calories less than the Du Bois value.

Reduced basal metabolic rates have also been found recently in New Orleans. Borgstrom and Hafkesburg (12) found the average basal metabolic rate of 9 trained subjects 18% lower than the Du Bois standard, 16% lower than the Harris and Benedict standard, and 14% lower than the Dreyer standard. Borgstrom does not give any explanation for his low metabolic rates, but suggests that among the factors concerned may be the warm climate and the lower protein intake. He found that protein intake was only 60% of the standard values given for New York.

Similar figures for protein intake have been obtained in Sydney. The results of determinations of blood urea concentration, urea excretion, and protein intake during the past few years in Sydney (Priestley and Hindmarsh, 30), have been much lower than the standard values quoted in text-books, and illustrate the danger of error in taking European and American standards as applicable here.

	Sydney.	America and England.
Blood urea concentration	11 mgm. urea N per 100 cc. blood	12 to 15 mgm.
Urea excretion	27·5 grms. urea in 24 hours ..	30 to 35 grms.
Protein intake	70 grms. in 24 hours	100 grms.

The climate of New Orleans is very similar to that of Sydney, although it is warmer.

From the results obtained in New Orleans, Brazil, and in Sydney, showing a reduction in basal metabolic rate of 18%, 24%, and 20% respectively from the Du Bois standard value, it would seem very probable that acclimatization to high external temperatures does take place, and that the body has the power of reducing its fundamental or basal metabolism to a lower level, thus facilitating the regulation of body temperature where heat loss is difficult.

However, the results of Wishart (37), which have appeared this year, on the basal metabolic rates of subjects in Glasgow, throw fresh light on the hypothesis of a reduction of metabolism as an acclimatization factor in the tropics. The basal metabolic rate of his subjects had been determined a number of times, so that they were trained subjects, and he found the average variation from the Du Bois standard of 3 out of 4 subjects to be —16%, —21·5%, and —21·3% respectively. Pickworth (29) has obtained similar results for the basal metabolic rates of subjects in Birmingham, namely, a reduction of about 20% from the Harris and Benedict predicted values. Pickworth explains his lower figures as due to the more complete muscular relaxation of his subjects than can be obtained by the use of bag methods, since the latter require the use of a mouthpiece or a face mask. Pickworth devised a small airtight chamber, and analyzed the air in it immediately before and a definite time after, usually 1 to 2 hours, the subject had rested quietly within it.

The average variations of the basal metabolic rates from the predicted values of Du Bois, found by the various workers in different parts of the world, and the mean external air temperatures, have been tabulated below.

Place.	Variation of B.M.R. From Du Bois.	Mean temperatures. 3 hottest months.	3 coldest months.	Yearly average.
Rio de Janeiro	—24%	24·2°C.	21·0°C.	22·6°C.
New Orleans	—18%	27·8°C.	13·3°C.	20·7°C.
Sydney (trained subjects)	—20%	21·7°C.	12·2°C.	17·2°C.
New York	—	22·2°C.	0°C.	11·3°C.
Birmingham	—20%	16·0°C.	5°C.	9·6°C.
Glasgow	—20%	11·7°C.	5°C.	9·0°C.

The lower figures for the basal metabolic rates obtained by the various workers mentioned in different parts of the world all show a reduction from the Du Bois standard of the same order. Except in the case of the Rio determinations all the experiments were carried out on trained laboratory subjects. This suggests that the lowered metabolic rates obtained were due to more complete muscular relaxation.

Pickworth claims that complete muscular relaxation cannot be obtained by the bag methods of determining basal metabolic rates, and that it is not possible for a fully trained subject to inhibit muscular tone, voluntary movements, and mental processes when using mouthpieces or face masks. He states, however, that when a subject is in the chamber for the first time a higher figure for metabolism is noted, which becomes less as the strangeness of the surroundings ceases to attract attention.

Subject 49 (see Table 4), when totally untrained in respiratory experiments, showed a progressive fall in his basal metabolic rate as he became used to the procedure and the apparatus; his results show that muscular relaxation is merely a matter of training and experience, and is not dependent on the type of apparatus used.

In 1876 Zuntz and Pfluger, from their curari experiments, showed that a decrease in muscular tone produced a diminution in the heat formation in muscular tissue, and as a result a fall in the total energy exchange of the body. In 1901 Frank and Voit showed that curari produced no diminution in the metabolism of an animal kept absolutely at rest. Thus the value obtained for the heat production of the body is affected by the state of muscular relaxation at the time of the experiment.

The basal metabolic rate obtained on a subject depends then, after all the standard conditions have been observed, on the degree of muscular relaxation acquired by the individual. In the cooler climates complete muscular relaxation requires practice to attain, but when it has been attained basal metabolic rates at least 20% lower than the Du Bois predicted values are obtained.

The average reduction in basal metabolic rate of 9% from the Du Bois value recorded in this investigation on 73 untrained subjects can then be explained as due to the fact that in the warmer climate of Sydney one relaxes more easily at rest, and in very hot climates, as in Brazil, the reduction of basal heat production by 24%, found by Almeida, points to a still more complete muscular relaxation. The feeling of lassitude in warm, humid climates, and the desire for movement and activity in low external temperatures are indications of the state of muscle tone. In warm-blooded animals body temperature is maintained at a constant level independent of climatic conditions, and the external mechanism for the regulation of body temperature is nervous. The action of cold on the skin stimulates peripheral nerve endings, and reflexly causes a greater heat production by increased tone of muscle, and at the same time reduces heat loss by vasoconstriction of the peripheral vessels. Exposure to heat, on the contrary, causes vasodilatation, and the production of sweat, and a lowering of muscle tone.

Increased metabolism can, of course, be induced without increase of muscular tone, as, for example, in fevers and hyperthyroidism.

The question as to how the organism adapts itself to high external temperatures is of considerable interest, and the lower basal metabolic rate that has been obtained on untrained subjects in warm and hot climates must be regarded as an acclimatization factor. It is suggested that this factor consists in the more ready production and maintenance of muscular relaxation, so that in climates where heat loss is more difficult there is, during rest, a lessened production of heat. The acquiring of this habit of relaxing readily and without conscious effort takes some time to become established in the body, and is more readily seen in individuals born in warm climates than in those who have migrated there after having adjusted their heat production to a cooler climate.

METHODS OF DETERMINING BASAL METABOLIC RATE.

The methods for the determination of the basal metabolic rate consist either in the measurement of heat loss, direct calorimetry, or in the measurement of heat production, indirect calorimetry. The close agreement between direct and indirect calorimetry has been definitely demonstrated by many workers, e.g., Rubner, Atwater and Benedict, and Du Bois. To measure directly the heat output of the body requires an elaborate and costly apparatus, a calorimeter, and since the essential factors in the formation of body heat are the processes of oxidation, heat production can be determined indirectly by the estimation of the oxygent intake or of the carbon dioxide output. Also Gephardt and Du Bois (21) have shown that because of the many errors in direct calorimetry it is advisable to use the method of indirect calorimetry for short and isolated experiments.

There are three methods in use for the determination of the basal metabolic rate by indirect calorimetry. The first method measures the litres of oxygen absorbed in a definite time, the second the weight of carbon dioxide expired in a definite time, and the third the volume of air expired in a definite time, and requires also the determination of its oxygen and carbon dioxide content.

The first method is the closed circuit or metabolimeter method of Benedict (5). The subject breathes into and out of a spirometer which has been filled with oxygen or air enriched with oxygen; the fall of the spirometer bell registers on a scale the amount of oxygen absorbed or the calorie production per hour. The respiratory quotient is assumed to equal 0·82. The carbon dioxide eliminated is absorbed by passing the expired air over soda-lime before it reaches the spirometer.

This method is very simple to carry out, but it has been greatly criticized by many workers. The main objection to the method seems to be the constant attention required to obtain reliable readings. A leak of only a few cubic centimetres, either in the apparatus or in the adjustment of the mask during the ten-minute determination, will appreciably affect the result, since the leak is measured as a loss of so much oxygen, and is not equivalent to the loss of so much air as in the gasometer method, thus increasing the error five times. The soda-lime also will need frequent changing, and it is difficult to gauge when a portion of the expired carbon-dioxide is escaping absorption. The danger of breathing contaminated air is great, the apparatus needs well sterilizing after each subject, and this is not readily done. This, with the constant checking required, make it difficult to use the metabolimeter for clinical work where many determinations are to be made. The possible error of this method has been estimated by Meakin and Davies (27) to equal 30%.

The second method is the gravimetric method of King (24). This is an open circuit method; the subject breathes through a mouthpiece or mask which has been fitted with an inlet and an outlet valve. Atmospheric air is inspired, and the expired air is passed through three jars containing calcium chloride, soda-lime, and calcium chloride respectively. The increase in the weight of the second and third bottles is a measure of the expired CO_2 in the time of the experiment. The heat production may be expressed as grammes of CO_2 per square metre per hour, or, using a constant R.Q. of 0·82, the heat production in calories per 24 hours can readily be calculated. This method is very simple to carry out, and does not require very elaborate apparatus. A very accurate balance is necessary, and large supplies of a high-grade soda-lime. King recommends a special soda-lime called Wilson soda-lime, which has greater absorbing powers for CO_2 than the usual soda-lime supplied for chemical work.

Wardlaw (36), in his modification of King's method, states that he uses 600 grms. of Merck's soda-lime: this with the jar will give a total weight of at least 1 kilogramme; the jar of calcium chloride will weigh a like amount. In six minutes, the time of his experiment, about 2 grms. of CO_2 are expired by an adult under basal conditions. This weight is divided between the second and third jars; thus the weighing machine is required to show an increase of perhaps less than 1 grm. in a kilogramme. A balance which can weight 1 kilogramme to 0·1 grm. accurately will give an error of at least 10% in the weighings, whereas if it can weigh 1 kilogramme to 0·01 grm. the error will equal 1%. A large balance with a high degree of accuracy is thus required. The checking of the efficiency of the soda-lime is important. To do this the expired air, after passing through the absorption bottles, is bubbled through lime-water or baryta-water. Wardlaw finds that it is necessary to renew the soda-lime after every ten determinations.

The heat production in calories per 24 hours can be calculated very simply, provided a constant R.Q. of 0·8185 is used instead of 0·82. The error produced by doing this is negligible, and a direct comparison of the heat production of the subject in calories per 24 hours with the predicted values of Du Bois and Harris and Benedict is made very easily. To do this the weight of CO_2 eliminated in 14·4 minutes is determined, and the figure obtained multiplied by 300 gives at once the metabolism of the subject in calories per 24 hours. When the R.Q. equals 0·82 the heat equivalent of 1 grm. of CO_2 is 2·99 calories. When the R.Q. equals 0·8185 the heat equivalent of 1 grm. of CO_2 is 3·00 calories, so that if 5 grms. of CO_2 are eliminated in 14·4 minutes the heat production of the subject is 5 × 300, or 1,500 calories per 24 hours.

The third method is the gasometer method already described. This is recognized by most workers as the most accurate method of determining the basal metabolic rate. Its chief drawback is the skill required in the technique of gas analysis.

A Comparison of the Results of the Three Methods.

From the oxygen and CO_2 content of the expired air of the subjects of this investigation, and the volumes of air expired, figures have been calculated to determine what results would have been obtained in each case in basal metabolic rate had the oxygen absorption alone been determined, or the CO_2 excretion alone, such as in the Benedict metabolimeter method or King's gravimetric method.

On the average slightly lower results were obtained with the oxygen absorption method and higher results by the gravimetric method than the gasometer method.

Average Variation from the Du Bois Standard.

			Range of Variation.	
Method.	Men.	Women.	Men.	Women.
Gasometer	—8·9%	—10·5%	+15·0% to —29·4%	+11·7% to —33·0%
O_2 absorption	—9·6%	—10·9%	+15·2% to —30·3%	+9·3% to —33·4%
CO_2 excretion	—6·0%	—7·3%	+15·4% to —25·1%	+21·6% to —35·9%

Range of Variation from the Mean B.M.R. as determined by each method.

Subject.	Gasometer Method.	O_2 Absorption Method.	CO_2 Excretion Method.
47 ..	—4·7% to +5·4%	—5·8% to +6·0%	—9·2% to +5·7%
X ..	—10·0% to +13·0%	—10·0% to +14·0%	—15·5% to +13·9%

These results differ from those of King, who claims that the gravimetric method gives lower figures for basal metabolic rates than either the oxygen absorption method or the gasometer method. He states that "If the basal metabolism is determined by a closed oxygen rich system, twice as many supernormal results may be expected among patients not clinically hyperthyroid as would be obtained by the gasometer method. Among the same group of cases more than twice as many supernormal results may be expected by the Tissot gasometer method as the measurement of CO_2 elimination." It is only fair to point out here that the determinations of basal metabolic rates on which King based the above conclusions were carried out on different groups of subjects and with different sets of apparatus, and one wonders if this is justifiable.

The figures in Tables 6 and 7, calculated for the comparison of the results which would be obtained by the three methods of determining basal metabolic rates by indirect calorimetry, were actually the same experiments, so that they represent the results on the same group of subjects and with the use of the same sets of apparatus throughout. This seems a fairer comparison.

The percentage variations of the abnormal results obtained by the three methods in this investigation are tabulated below. A variation of ±10% was taken as the normal range. The variation was calculated from the average variation of all the subjects from the Du Bois standard obtained by each method. Thus in the gasometer method the normal range was considered to be ±10% from a standard 8·9% below Du Bois for men and 10·5% below the Du Bois standard for women.

Method.	% Abnormal Results.	% Supernormal Results.	% Hyponormal Results.
Gasometer	18%	11%	7%
O_2 absorption ..	18%	11%	7%
CO_2 excretion ..	19%	11%	8%

These results show that the same number of abnormal results should be obtained in the basal metabolic rate determinations by all three methods. Differences then that are obtained must be due to errors in technique of the method, and if lower results are obtained by the gravimetric method, then the whole of the CO_2 expired has not been taken up by the soda-lime.

It became evident while carrying out this investigation that often the first basal metabolic rate determination was higher than the succeeding ones, and that this was associated with a higher volume output of air than normal and a high R.Q. The CO_2 excretion method in these cases gave much higher results than the O_2 absorption methods. (These results are marked with an asterisk in Tables 1 and 2.)

King claims that the analysis of 157 published observations on gas exchange showed that CO_2 is either not washed out during the practical application of the basal metabolic rate test, or else it is washed out in negligible amounts.

Boothy and Sandiford (9) point out that it is important to impress upon patients the necessity of breathing naturally. The tendency of patients to vary the depth and regularity of their respirations during the tests does not affect the total metabolism materially, but it does affect the R.Q. Abnormal R.Q.'s above 1·00 or below 0·7 indicate as a rule a nervous condition of the patient or an error in technique. They state that it is advisable to repeat such cases, although they have rarely found a sufficient change in the check determination to affect the clinical value of the report. In this investigation it was found that irregular respiration, affecting as it does the lung ventilation of the subject, may affect the metabolism figure obtained appreciably.

Some experiments were then carried out to study the effect of increasing lung ventilation. The increase in the rate of respiration and the increase in the depth were taken separately. The results are given in Tables 8 and 9, and it is very evident from them that increased lung ventilation causes a marked rise in the metabolism figure obtained in the R.Q. The latter is due to the fact that increased lung ventilation washes out CO_2 at a greater rate than it increases the intake of oxygen.

Table 11 gives a summary of the results of these experiments, and shows that increased lung ventilation by increased depth affects the basal metabolic rate determination to a greater extent than increased rate, and by both processes the metabolism figure obtained is markedly increased.

The increased elimination of CO_2 obtained by increased rate of respiration does not agree with the results of Lossen and quoted by Lusk. Lossen found that the CO_2 elimination was independent of the ventilation of the lungs.

When the basal metabolism rate determination is calculated from the CO_2 elimination alone the increase in the figure obtained after increasing lung ventilation is much greater than when calculated on the oxygen intake alone or by using the oxygen intake and the variable R.Q.

King (25) claims that the CO_2 output is less variable than the oxygen intake, and that CO_2 elimination is possibly a more accurate index to heat production than is oxygen consumption. The experiments on which these conclusions are based were continued for a period of one hour, and this is not possible with patients in general for indirect calorimetry. Errors would at once appear, due to restlessness. In short period experiments, 10 to 15 minutes' duration, a subject is much more likely to increase the output of CO_2 than in an experiment of one hour, for in the latter there would be time to compensate for the early washing out of CO_2 by a later retention.

When the R.Q. equals 0·82 no difference is obtained in the basal metabolic rate, whether determined by the oxygen absorbed, CO_2 output, or the gasometer method, but in subjects who show an appreciable variation in the volume output of expired air the basal metabolic rates recorded by the CO_2 method are much higher than those determined by the oxygen absorption or the gasometer methods.

Normal subjects, in the strict basal conditions required for a basal metabolic rate determination, have an R.Q. of 0 82, and any increase in this indicates a high basal metabolic rate determination, due to increased lung ventilation or to nervous stimulation. This suggests then that a basal metabolic rate determination which has an R.Q. of over 0·9 should be repeated, and perhaps discarded. The range of variation for normal metabolic rates is such that small increases in the lung ventilation or slight nervous stimulation are not of importance clinically. Occasionally one finds a subject who will over-breathe, even when the test has been repeated three times, and it is well to realize that as a basal metabolic rate determination the results are useless. Subject (a), Table 1, is an example of this.

The experiments which recorded R.Q.'s approaching 1·00, and where the volume of air expired was appreciably greater than the other tests on the same subject, were not used in calculating the average basal metabolic rate of the subjects in Tables 1 and 2.

A high R.Q. is not always associated with a high volume output of expired air, but where it was it seemed advisable to discard the results.

In determining the basal metabolic rate then by the gasometer method one has the advantage of checking the test in this way, for an R.Q. of over 0·9 arouses suspicion of faulty technique or the collection of an abnormal sample. The results obtained on subject 49 are of interest here. The basal metabolic rate was determined for 5 consecutive days; the first three days recorded a consistent fall in the volume of expired air, the R.Q., and the basal metabolic rate. In the first experiment the basal metabolic rate determined from the CO_2 excretion gave a figure 140 calories higher than the gasometer method, and 170 calories higher than the oxygen absorption method, but in the later experiments, when a sample of air more nearly basal was collected, there were no appreciable differences in the figures obtained by the three methods.

Except for gross manifestations like movement, excitement, etc., there is no method of gauging whether a true basal metabolic rate is being determined or not, since one cannot estimate the degree of muscular relaxation and the psychological condition of the subject.

One realizes that a high basal metabolic rate in normal subjects may be due not only to increased lung ventilation, the nervous factor is a large and important one in a number of cases, but the fact that increases in lung ventilation can so easily be obtained, and cause a high figure to be given for basal metabolic rate, is important, especially since breathing through a mask or mouthpiece tends of itself to increase lung ventilation.

The normal range of variation of the volume output of expired air from 21 experiments on subject X was —19% to +10% from the average, and for 12 experiments on subject 50 from —18% to +14%. Thus a normal range of variation in lung ventilation was found to be about 30% in trained subjects.

Moderate increases in the lung ventilation may show appreciable increases in the basal metabolic rate.

Increase in Lung Ventilation.	Increase in B.M.R. Gasometer Method.	Increase in B.M.R. CO_2 Excretion Method.	Effect on R.Q.
32%	7%	10%	0·85 to 0·85
39%	9%	—9%	0·85 to 0·67
48%	22%	36%	0·84 to 0·97
52%	14%	31%	0·82 to 0·99
56%	19%	43%	0·90 to 1·11

The contention that in a 15-minute collection of expired air the increased lung ventilation will only continue for the first 5 minutes, and that during the next 10 minutes any washing out of CO_2 will be compensated by a retention of CO_2 is not borne out by the results of some experiments on voluntarily increasing lung ventilation.

The results in Table 12 show that in an experiment where increased lung ventilation was maintained for 15 minutes, and the samples of air were collected at 5-minute intervals, more CO_2 was eliminated in the last 5 minutes than in the first or second samples. The subject did not find it difficult to maintain this increased breathing, nor suffer from any after-effects. In the other similar experiments (Tables, 11, 13, and 14), although the CO_2 excretion fell steadily in the three 5-minute samples of air, the third sample contained over 50% more CO_2 than normal for the subject. These experiments show very exaggerated conditions of superventilation, still one sometimes collects 100 litres of air from a subject, and it is well to recognize that these increased lung ventilations can be maintained quite easily. If the subject is inclined to over-breathe, it is better to discard the figures and repeat the tests at a future date.

If over-ventilation of the lungs takes place to any extent a normal sample of expired air cannot be obtained for at least half an hour afterwards, for it takes some time for the alveolar CO_2 to reach its normal level again. (Table 8.)

With subject X the normal basal metabolic rate was 1,158 calories per 24 hours. On increasing lung ventilation from 50·8 litres to 156 litres in 14·4 minutes the metabolic rate obtained was 1,494 calories; a quarter of an hour later the normally expired air sample gave a metabolism figure of 866 calories per 24 hours, with a volume output of air of only 36·8 litres.

A determination of the composition of alveolar air, obtained by collecting the last portion of a deep expiration, was carried out. No alteration in the alveolar CO_2 and oxygen content was obtained before and after giving a sample of expired air for 14·4 minutes into a Douglas bag. It was found that after forced breathing the alveolar oxygen had increased from 14·42% to 17·19%, and the alveolar CO_2 had decreased from 5·47% to 3·66%. This means that at the end of 14·4 minutes increased lung ventilation there was present in the body (mostly in the lungs, and to a lesser extent in the blood) an extra 122 cc. oxygen. This is measured as oxygen absorption, so an increase in the metabolism figure is obtained. Similarly the washing out of 1,430 cc. of extra CO_2 is measured as CO_2 production by the body during the experiment.

The collection of the last portion of a deep expiration after holding the breath for 30 seconds was next carried out, as it was hoped by this means to obtain some idea of the amount of oxygen and CO_2 in the venous blood after normal respirations and after superventilation of the lungs. The results are given in Tables 11, 13, and 14, but the figures obtained were not constant enough to enable one to calculate the actual amount of increase in the venous oxygen tension or the lowering of the CO_2 venous tension.

The CO_2 output and the oxygen intake as determined by the Douglas bag sample, may not be a true index of the CO_2 produced and of the oxygen absorbed by the body in the time of the test. The normality of the sample of expired air collected may be tested by a determination of the alveolar CO_2 and oxygen of the subject immediately before the collection of the Douglas bag sample, and again immediately after the sample has been collected. If the alveolar oxygen percentage is higher, and the CO_2 percentage is lower in the second sample of alveolar air, then it is obvious that the subject has over-ventilated the lungs during the test.

In the gasometer method of determining the basal metabolic rate one has a check on the normality of the sample collected from the volume of the expired air and the R.Q. If there is still some doubt then, in subsequent estimations, the determination of the oxygen and CO_2 content of the alveolar air of the subject immediately before and immediately after the collection of the sample gives definite information as to whether over-ventilation has or has not taken place.

In conclusion, I wish to express my thanks to Professor H. G. Chapman, in whose laboratory this work was carried out, for his advice, and also to Associate Professor Priestley for his helpful criticism.

SUMMARY.

1. The basal metabolic rate of 76 subjects was determined by the gasometer method. An average reduction of 8·9% was obtained for the men and 10·5% for the women when compared with the Du Bois predicated values.

2. It is suggested that the reduced basal metabolic rate is due to the more ready muscular relaxation of the subjects in the warmer climate of Sydney.

3. Basal metabolic rate determinations from oxygen absorption alone, using a fixed R.Q., showed an average reduction of 9·6% for men and 10·9% for women when compared with Du Bois predicted values.

4. Basal metabolic determinations from CO_2 output alone, with a fixed R.Q., showed an average reduction of 6·0% for men and 7·3% for women.

5. The gasometric method, where the oxygen absorbed, the CO_2 excreted, and the respiratory quotient are all determined, should give the most consistent results for basal metabolic rate determinations.

6. The determination of R.Q. and volume output of expired air is a useful check on the normal breathing of a subject.

7. Determinations of alveolar CO_2 and oxygen immediately before and immediately after the test give the most accurate information of the normality of the sample collected.

TABLE 1. Women.

Subject	Age Years	Surface Area Sq. M.	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in Litres 14.4 Minutes	% Oxygen Absorbed	R.Q.	Calories per 24 Hours. Basal Metabolism Gasometer Method	Average B.M.	Du Bois Standard	Harris and Benedict Standard	% Variation of Average from Du Bois	Harris and Benedict	Calories per Sq. M. per hour
A	19	1·52	67	98·3°F.	—	71·0	3·55	0·932	1252	1268	1386	1341	—8·4	—5·4	34·8
			70	98·5°F.	—	69·1	3·99	0·779	1284						
B	19	1.55	65·68	98·4°F.	18	79·3	3·68	0·827	1408	1309	1414	1370	—7·4	—4·5	35·2
			65·65	98·6°F.	18	61·4	4·01	0·773	1175						
			62·64	98·0°F.	—	66·9	4·11	0·783	1313						
			60	98·0°F.	14	69·0	4·03	0.811	1338						
C	19	1·72	—	—	—	68·8	4·11	0·849	1374	1288	1569	1485	—18·5	—13·9	31·0
			73	—	—	76·6	3·26	1·01	1260*						
			—	—	—	65·0	3·87	0·778	1202						
D	20	1·42	—	—	—	88·5	3·15	0·914	1381	1428	1278	1296	+11·7	+10·2	41·9
			—	—	—	79·8	3·80	0·871	1478						
			69	—	—	79·0	3·64	0·923	1424						
E	20	1·43	78	98·2°F.	—	73·6	3·38	0·985	1251*	1	1287	1302	—12·6	—13·5	34·0
			74	98·7°F.	14	61·4	3·65	0·871	1095	1126					
			—	98·5°F.	—	55·0	4·34	0·838	1158						
F	20	1·45	74	98·2°F.	—	48·7	4·63	0·931	1120	1200	1305	1317	—8·0	—8·9	34·5
			72	98·3°F.	12	56·8	4·61	0·874	1281						
G	20	1·48	60	98·2°F.	—	62·7	3·70	0·870	1135	1169	1332	1344	—12·2	—13·0	32·9
			60	98·1°F.	12	55·2	4·51	0·829	1204						
H	20	1·59	—	98·8°F.	16	66·6	3·63	0·928	1198	1227	1431	1375	—14·3	—10·8	32·2
			—	—	—	63·6	3·99	0·922	1256						
I	20	1·61	60·62	98·4°F.	18	74·5	3·94	0·817	1419	1305	1448	1451	—9·9	—10·1	33·8
			60·62	98·0°F.	18	72·1	3·51	0·897	1246						
			60·60	98·5°F.	18	70·3	3·71	0·787	1249						

TABLE 1. Women (continued).

									Calories per 24 Hours.				% Variation of Average from		
Subject	Age Years	Surface Area Sq. M.	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in Litres 14.4 Minutes	% Oxygen Absorbed	R.Q.	Basal Metabolism Gasometer Method	Average B.M.	Du Bois Standard	Harris and Benedict Standard	Du Bois	Harris and Benedict	Calories per Sq. M. per hour
J	21	1·42	—	—	—	80·1	3·48	0·744	1320*	—	1261	1274			
			—	—	—	99·7	2·37	0·962	1181*						
			—	—	—	93·0	2·18	1·01	1023*						
K	21	1·50	—	—	—	54·4	4·34	0·829	1141	1128	1331	1337	—15·2	—15·6	31·3
			—	—	—	57·8	3·94	0·883	1116						
L	21	1·54	76	98·2°F.	—	69·1	3·49	1·03	1217	1220	1367	1347	—10·8	—9·4	33·0
			80	98·8°F.	—	68·4	3·63	0·900	1222						
M	21	1·57	69·69	98·6°F.	17	63·3	4·59	0·756	1377	1336	1394	1367	—4·2	—2·3	35·5
			68·67	98·6°F.	12	61·6	4·19	0·753	1294						
N	21	1·59	—	98·0°F.	—	62·2	3·44	0·816	1031	1238	1411	1364	—12·3	—9·2	32·5
			70	98·4°F.	—	76·3	3·83	0·812	1407						
			—	—	—	74·4	3·51	0·866	1276						
O	21	1·76	54	—	—	55·5	5·29	0·796	1409	1396	1562	1516	—10·6	—7·9	33·1
			—	—	—	59·3	4·84	0·812	1382						
P	21	1·65	64	98·2°F.	—	63·7	4·60	0·809	1409	1215	1465	1433	—17·1	—15·2	30·7
			—	—	—	63·8	3·76	0·849	1167						
			75	—	—	51·5	4·28	0·836	1069						
Q	22	1·62	79·73	98·6°F.	17	65·7	4·22	0·805	1333	1335	1438	1400	—7·2	—4·6	34·3
			70·78	98·3°F.	18	70·7	3·98	0·759	1337						
R	22	1·76	60·58	98·7°F.	20	76·2	3·70	0·770	1344	1353	1563	1490	—13·4	—9·2	32·0
			59·64	98·4°F.	17	74·0	3·83	0·804	1362						
S	23	1·52	66·67	98·6°F.	14	65·3	3·92	0·824	1236	1255	1350	1335	—7·0	—6·0	34·4
			60	—	—	69·6	3·58	0·981	1258						
			69·70	98·0°F.	16	63·1	4·11	0·883	1272						

TABLE 1. Women (continued).

Subject	Age Years	Surface Area Sq. M.	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in Litres 14.4 Minutes	% Oxygen Absorbed	R.Q.	Calories per 24 Hours.				% Variation of Average from		Calories per Sq. M. per hour per hour
									Basal Metabolism Gasometer Method	Average B.M.	Du Bois Standard	Harris and Benedict Standard	Du Bois	Harris and Benedict	
T	24	1·80	—	—	—	63·4	3·50	0·857	1081	1056	1598	1538	—33·8	—31·3	24·5
			60	97·1°F.	20	64·6	3·13	0·817	975						
			56	97·2°F.	—	64·1	3·65	0·671	1112						
U	25	1·40	70	98·8°F.	—	48·1	4·97	0·791	1145	1145	1243	1255	—7·9	—8·8	34·1
V	26	1·62	84	98·0°F.	12	61·7	5·08	0·752	1487	1393	1438	1425	—3·1	—2·2	35·8
			78·79	98·0°F.	12	65·4	4·75	0·760	1476						
			67·69	98·1°F.	12	59·1	4·45	0·881	1291						
			67·67	98·4°F.	12	62·6	4·37	0·821	1319						
W	40	1·39	68·70	98·7°F.	12	52·2	4·23	0·825	1066	1066	1209	1162	—11·8	—8·3	32·0
X	32	1·46	—	—	—	49·8	4·36	0·824	—	1046	1297	1238	—19·5	—15·5	29·8
Y	40	1·43	72·73	98·0°F.	6	46·7	5·19	0·815	1170	1149	1244	1176	—7·6	—2·3	33·5
			70·70	98·0°F.	6	49·4	4·73	0·818	1128						
Z	56	1·68	—	—	—	69·7	4·13	0·871	1407	1407	1411	1359	—0·3	+3·5	34·9
a	12	1·52	73	99·1°F.	22 23 26	97·1	3·32	0·904	1589	1589	1641	—	—3·2	—	43·5
			64	98·9°F.	26	168·0	2·12	1·29	1797*						
			—	98·9°F.	20	158·6	2·37	1·28	1896*						
Averages								0·834					—10·5	—8·6	
Standard Deviations . .													22·1	18·7	
Probable Errors of the Averages													±2·92	±2·52	

*Abnormal results not considered in averages. See text.

TABLE 2. Men.

Subject	Age Years	Surface Area Sq. M.	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in Litres 14.4 Minutes	% Oxygen Absorbed	R.Q.	Calories per 24 Hours. Basal Metabolism Gasometer Method	Calories per 24 Hours. Average B.M.	Calories per 24 Hours. Du Bois Standard	Calories per 24 Hours. Harris and Benedict Standard	% Variation of Average from Du Bois	% Variation of Average from Harris and Benedict	Calories per Sq. M.
1	19	1·61	—	—	12	68·8	4·89	0·818	1623	1675	1584	1524	+6·2	+9·9	43·4
			—	—	9	56·9	6·33	0·687	1677						
			—	98·6°F.	6	62·6	5·81	0·762	1725						
2	19	1·62	70	98·4°F.	—	53·3	4·33	0·965	1155	1155	1594	1545	—27·5	—24·6	29·7
			60	98·4°F.	—	74·0	3·56	0·991	1326*						
			52	98·8°F.	—	65·6	3·30	1·02	1091*						
3	19	1·77	—	—	14	72·3	4·27	0·866	1508	1510	1742	1678	—13·3	—10·0	35·6
			—	—	12	68·5	4·64	0·827	1536						
			—	98·2°F.	11	70·0	4·33	0·879	1486						
4	19	1·78	—	—	—	69·9	4·53	0·984	1592	1605	1751	1708	—8·3	—6·0	37·5
			—	—	—	76·6	4·33	0·889	1630						
			—	—	—	61·3	5·44	0·783	1594						
5	19	1·79	—	—	—	71·0	4·78	0·790	1624	1577	1761	1680	—10·5	—6·1	36·7
			—	98·4°F.	14	68·0	4·91	0·798	1601						
			—	98·4°F.	16	72·8	4·27	0·838	1507						
6	19	1·94	64	97·8°F.	14	77·5	4·29	0·843	1613	1613	1909	1849	—14·4	—12·8	34·6
7	19	1·94	—	—	—	83·4	4·52	0·973	1886	1977	1824	1839	+8·4	+7·5	42·4
			—	—	—	83·6	4·99	0·930	2069						
8	19	1·68	67	98·8°F.	—	61·6	3·97	0·826	1181	1182	1653	1585	—28·4	—25·4	29·3
			66	98·8°F.	—	69·4	3·47	0·890	1183						
9	20	1·65	—	—	—	78·9	4·00	0·872	1543	1532	1584	1575	—3·3	—2·7	38·7
			—	—	—	68·9	4·64	0·769	1522						
10	20	1·67	—	—	—	77·7	3·55	0·927	1366	1426	1603	1569	—11·0	—9·1	35·3
			—	—	—	84·9	3·60	1·06	1546*						
			—	—	—	71·4	4·25	0·875	1486						

TABLE 2. Men (continued).

Subject	Age Years	Surface Area Sq. M.	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in Litres 14.4 Minutes	% Oxygen Absorbed	R.Q.	Calories per 24 Hours.				% Variation of Average from		Calories per Sq. M. per hour
									Basal Metabolism Gasometer Method	Average B.M.	Du Bois Standard	Harris and Benedict Standard	Du Bois	Harris and Benedict	
11	20	1·68	—	—	10	52·0	4·82	0·912	1236	1322	1612	1581	—18·0	—16·4	32·8
			—	—	9	51·5	5·19	0·807	1286						
			—	—	8	57·8	5·15	0·844	1444						
12	20	1·68	—	—	11	66·2	4·20	0·885	1364	1418	1613	1585	—12·1	—10·5	35·2
			—	98·0°F.	14	71·4	4·25	0·856	1471						
13	20	1·69	—	—	—	69·7	3·88	0·951	1347	1442	1622	1588	—11·1	—9·2	35·6
			—	—	—	·69·5	4·53	0·905	1553						
			—	—	—	62·9	4·58	0·921	1425						
14	20	1·71	72·69	98·2°F.	—	91·2	3·39	0·908	1526*	—	1642	1618	—12·6	—11·4	34·9
			66	98·4°F.	17	109·2	2·71	1·19	1499*						
			68·67	98·0°F.	—	67·3	4·43	0·821	1433	1433					
15	20	1·75	—	—	—	64·4	4·33	0·888	1370	1393	1680	1676	—17·1	—16·8	33·2
			—	—	—	64·4	4·49	0·875	1416						
16	20	1·75	—	—	—	85·9	3·79	0·881	1594	1497	1680	1679	—10·9	— 10·8	35·7
			—	—	—	83·9	3·60	0·927	1496						
			—	—	—	68·9	4·20	0·828	1402						
17	20	1·75	—	—	11	63·2	4·93	0·850	1515	1432	1680	1637	—14·8	—12·5	34·1
			—	—	12	60·8	4·44	0·952	1348						
18	20	1·76	—	—	12	70·5	4·33	0·794	1464	1541	1690	1665	—8·8	—7·4	36·5
			—	—	12	77·6	4·32	0·826	1619						
			—	—	15	81·4	3·88	0·866	1540						
19	20	1·78	—	—	10	69·1	4·91	0·876	1661	1678	1709	1689	—1·8	—0·7	39·3
			—	—	12	70·0	4·94	0·902	1704						
			76·64	98·3°F.	12	68·4	5·04	0·837	1670						
20	20	1·79	—	—	14	78·7	4·55	0·835	1734	1658	1719	1718	—3·5	—3·5	38·6
			—	—	17	75·8	4·45	0·840	1635						
			—	—	—	72·8	4·57	0·822	1606						

TABLE 2. Men (continued).

Subject	Age Years	Surface Area Sq. M.	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in Litres 14.4 Minutes	% Oxygen Absorbed	R.Q.	Calories per 24 Hours. Basal Metabolism Gasometer Method	Average B.M.	Du Bois Standard	Harris and Benedict Standard	% Variation of Average from Du Bois	Harris and Benedict	Calories per Sq. M. per hour
21	20	1·82	—	98·6°F.	11	66·6	4·63	0·861	1504	1523	1747	1726	—12·8	—11·8	34·9
			—	98·4°F.	10	64·2	4·90	0·876	1541						
22	20	1·87	70	—	—	72·1	4·05	0·911	1442	1537	1795	1804	—14·4	—14·8	34·1
			64	94·4°F.	—	81·9	4·08	0·863	1631						
23	20	1·96	65	—	—	65·0	5·04	0·795	1571	1507	1882	1868	—19·9	—19·4	32·0
			60	—	—	67·7	4·43	0·817	1442						
			—	—	—	78·4	3·89	0·913	1507						
24	20	1·90	—	—	—	80·6	5·02	0·836	1956	1848	1824	1839	+1·3	+0·5	40·5
			—	—	—	74·4	4·79	0·866	1740						
			—	—	—	107·6	3·89	1·01	2113*						
25	21	1·62	—	—	15	79·9	3·64	0·975	1460	1493	1536	1507	—2·8	—0·9	38·4
			—	98·9°F.	19	84·1	3·68	0·708	1525						
26	21	1·67	70·70	98·2°F.	11	66·2	5·13	0·850	1652	1649	1583	1574	+4·2	+4·7	41·2
			69·68	98·2°F.	10	66·3	5·00	0·836	1606						
			69·70	98·3°F.	12	68·9	4·90	0·844	1640						
27	21	1·72	—	—	—	71·1	4·32	0·896	1511	1511	1630	1617	—7·3	—6·5	36·6
			—	—	—	82·4	3·99	0·990	1655*						
			—	—	—	89·2	3·96	0·944	1759*						
28	21	1·72	—	98·6°F.	8	72·6	4·55	0·824	1596	1619	1630	1647	—0·7	—1·7	39·2
			—	98·3°F.	14	72·2	4·75	0·884	1682						
			—	98·7°F.	8	66·2	4·88	0·869	1579						
29*	21	1·73	—	—	18	73·3	3·89	0·897	1401	1428	1640	1622	—12·9	—12·0	34·4
			80	98·7°F.	16	68·4	4·64	0·756	1508						
			82·82	98·6°F.	15	70·0	3·99	0·899	1375						
30	21	1·74	—	—	—	80·6	3·82	1·01	1554*	1561	1649	1628	—5·5	—4·3	37·3
			—	—	—	72·1	4·35	0·944	1561						

TABLE 2. Men (continued).

Subject *	Age Years	Surface Area Sq. M.	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in Litres 14.4 Minutes	% Oxygen Absorbed	R.Q.	Calories per 24 Hours. Basal Metabolism Gasometer Method	Average B.M.	Du Bois Standard	Harris and Benedict Standard	% Variation of Average from Du Bois	Harris and Benedict	Calories per Sq. M. per hour
31	21	1·78	78	—	—	80·2	4·51	0·851	1758	1804	1687	1670	+6·9	+8·0	42·2
			78·76	99·0°F.	12	78·5	4·60	0·865	1763						
			78	99·0°F.	12	81·0	4·82	0·836	1892						
32	21	1·79	—	98·2°F.	14	76·8	4·41	0·789	1622	1543	1697	1686	—9·1	—8·5	35·9
			—	98·4°F.	9	60·1	5·23	0·835	1521						
			68	98·6°F.	—	61·8	4·86	0·913	1485						
33	21	1·77	—	—	10	59·2	5·10	0·845	1467	1415	1678	1688	—15·7	—16·2	33·3
			—	—	9	51·2	5·59	0·772	1362						
34	21	1·93	—	98·5°F.	10	95·7	3·70	1·07	1787*	—	1830	1822			
			—	98·5°F.	7	71·5	5·11	0·812	1759	1684	—	—	—8·0	—7·5	36·4
			—	98·4°F.	8	69·1	4·75	0·884	1609						
35	21	1·70	—	—	—	88·8	4·01	1·05	1797*	1632	1611	1590	+1·3	+2·4	40·0
			—	—	—	74·7	4·97	0·913	1834*						
			—	—	—	72·9	4·50	0·940	1632						
36	21	1·96	—	—	—	81·3	5·07	0·879	2020	1982	1858	1891	+6·7	+4·8	42·1
			—	—	8	78·9	4·95	0·854	1901						
			—	—	12	80·5	5·15	0·864	2024						
37	21	2·04	74	—	—	76·2	43·0	1·01	1652*						
			68	—	—	66·3	52·3	0·871	1695	1695	1934	1987	—12·4	—14·7	34·6
38	22	1·74	—	—	—	63·0	4·56	0·820	1385	1364	1649	1625	—17·3	—16·1	32·7
			—	98·4°F.	12	64·8	4·82	0·784	1493						
			—	—	—	59·0	4·16	0·911	1213						
39	22	1·85	74	98·2°F.	10	63·6	5·13	0·760	1550	1536	1754	1733	—12·4	—11·4	34·6
			74	97·9°F.	14	68·6	4·89	0·730	1584						
			72	98·5°F.	—	71·6	4·29	0·800	1475						
40	22	1·85	—	98·0°F.	9	64·8	4·64	0·808	1447	1498	1754	1745	—14·6	—14·2	33·8
			—	98·1°F.	8	72·1	4·46	0·820	1550						

TABLE 2. Men (continued).

Subject	Age Years	Surface Area Sq. M.	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in Litres 14·4 Minutes	% Oxygen Absorbed	R.Q.	Calories per 24 Hours. Basal Metabolism Gasometer Method	Average B.M.	Du Bois Standard	Harris and Benedict Standard	% Variation of Average from Du Bois	Harris and Benedict	Calories per Sq. M. per hour
41	22	1·93	—	—	10	78·2	5·50	0·805	2065	2105	1830	1843	+15·0	+14·2	45·4
			—	—	10	77·6	5·76	0·779	2134						
			—	—	10	80·9	5·37	0·862	2115						
42	22	1·65	—	—	—	71·3	4·76	0·842	1646	1596	1564	1539	+2·0	+3·7	40·3
			65	—	—	75·9	4·26	0·782	1545						
43	23	1·63	—	97·9°F.	12	67·2	4·13	0·934	1378	1320	1545	1516	—14·5	—12·9	33·8
			72	98·1°F.	12	67·6	3·94	0·934	1322						
			69	—	—	54·0	5·20	0·742	1327						
			—	—	—	63·3	4·01	0·902	1251						
44	23	1·65	—	98·1°F.	12	68·7	4·64	0·763	1516	1512	1564	1554	—3·3	—2·7	38·2
			—	98·5°F.	11	69·5	4·53	0·794	1508						
45	25	1·63	73	98·6°F.	16	70·0	4·64	0·836	1574	1587	1545	1504	+2·7	+5·5	40·6
			—	98·0°F.	—	82·2	3·96	0·894	1600						
46	25	1·86	60	97·9°F.	—	74·3	3·38	1·04	1267*						
			56	98·0°F.	—	63·7	4·00	0·872	1245	1245	1763	1752	—29·4	—28·9	27·8
47	26	1·55	—	—	—	75·6	3·71	0·911	1385	1476	1469	1425	+0·2	+3·6	39·7
			—	—	—	79·0	3·96	0·856	1524						
			—	—	—	79·5	4·04	0·829	1518						
48	27	1·85	—	98·1°F.	8	71·0	4·87	0·903	1703	1746	1754	1713	—0·1	+2·1	39·3
			—	98·2°F.	7	59·4	6·28	0·794	1789						
49	27	1·63	—	—	—	50·7	4·97	0·788	—	1198	1545	1468	—22·4	—18·4	30·5
50	42	1·85	—	—	—	75·7	3·84	0·836	—	1408	1710	1599	—18·0	—12·3	31·7
				Averages . .			. .	0·850					—8·9	—7·6	
									Standard Deviations . .				19·9	17·6	
									Probable Errors of the Averages . .				±1·89	±1·68	

*Abnormal results not considered in averages. See text.

TABLE 3. Subject X.

Date	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in 14.4 Minutes	% Oxygen Absorbed	% CO_2 Expired	R.Q.	Basal Metabolism Gasometer Method	O_2 Method	CO_2 Method	Du Bois Standard	Harris and Benedict Standard
7-25	—	—	—	49·8	4·20	3·72	0·878	1024	1009	1091	1297	1238
7-25	—	—	—	50·0	4·31	3·24	0·745	1021	1040	956		
7-25	—	—	—	50·7	4·07	3·33	0·811	994	996	996		
7-25	—	—	—	54·3	4·23	3·35	0·784	1098	1108	1072		
7-25	—	—	—	48·2	4·30	3·15	0·726	976	1000	895		
8-25	—	—	—	53·3	3·81	3·34	0·869	992	980	1049		
8-25	—	—	—	54·8	3·83	3·35	0·867	1025	1013	1081		
23- 9-25	80	—	—	53·0	3·81	3·34	0·869	987	974	1043		
30- 9-25	78	—	—	50·3	3·83	3·35	0·867	940	930	993		
7-10-25	78	—	—	51·3	4·60	3·91	0·843	1146	1138	1182		
15-12-25	—	—	11	40·2	5·09	3·93	0·766	974	987	931		
2-26	78	97·5°F.	12	53·2	4·34	3·58	0·818	1112	1114	1122		
16- 6-26	80	98·6°F.	10	49·0	4·63	3·78	0·809	1092	1095	1092		
18-11-26	77	98·7°F.	10	50·8	4·77	3·75	0·779	1158	1167	1120		
25-11-26	69	98·5°F.	10	46·0	4·56	3·90	0·848	1020	1015	1061		
30-11-26	69	98·6°F.	9	44·7	4·78	4·05	0·841	1036	1031	1067		
1-12-26	74	99·0°F.	10	51·4	4·80	3·94	0·814	1189	1190	1194		
5- 2-27	72	98·0°F.	10	48·8	4·40	3·98	0·900	1057	1036	1145		
7- 2-27	70	97·7°F.	10	50·2	4·22	3·59	0·844	1031	1022	1062		
2- 3-27	68	—	9	47·6	4·44	3·59	0·801	1017	1020	1009		
9- 3-27	71	—	9	47·8	4·67	3·87	0·822	1075	1077	1088		
	Average	..	..	49·8	4·36	3·62	0·824	1046	1045	1059		

TABLE 4. Subject 49.

Date	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in 14·4 Minutes	% Oxygen Absorbed	% CO_2 Expired	R.Q.	Basal Metabolism Gasometer Method	Basal Metabolism O_2 Method	Basal Metabolism CO_2 Method	Du Bois Standard	Harris and Benedict Standard
9-5-27	—	—	20	83·9	3·66	3·35	0·907	1515	1482	1657	1545	1468
10-5-27	65	—	16	60·0	4·49	3·67	0·811	1298	1300	1298		
11-5-27	60	98·7°F.	11	50·9	5·04	3·98	0·784	1226	1238	1197		
12-5-27	58	98·6°F.	9	45·9	5·38	4·24	0·783	1180	1191	1147		
13-5-27	59	98·2°F.	14	55·2	4·49	3·61	0·797	1188	1196	1174		
	Average	..	..	50·7	4·97	3·94	0·788	1198	1208	1173		

TABLE 5. Subject 50.

Date	Pulse	Mouth Temperature	Respiration Rate	Volume Expired in 14·4 Minutes	% Oxygen Absorbed	% CO_2 Expired	R.Q.	Basal Metabolism Gasometer Method	Basal Metabolism O_2 Method	Basal Metabolism CO_2 Method	Du Bois Standard	Harris and Benedict Standard
7-25	—	—	—	78·7	3·63	3·10	0·846	1386	1378	1437	1710	1599
7-25	—	—	—	62·4	4·61	3·55	0·763	1367	1388	1306		
8-25	—	—	—	77·8	3·87	3·18	0·814	1451	1453	1458		
8-25	76	—	—	76·7	3·78	2·97	0·777	1383	1399	1343		
25- 2-26	—	—	—	76·8	3·55	3·32	0·926	1352	1316	1503		
4- 3-26	—	—	—	72·8	3·77	3·32	0·873	1342	1324	1416		
23-11-26	67	98·4°F.	12	71·6	4·02	3·43	0·846	1399	1390	1451		
26-11-26	67	98·0°F.	10	78·2	3·66	3·26	0·882	1403	1383	1503		
2-12-26	58	98·0°F.	10	76·9	3·72	3·34	0·889	1404	1383	1511		
7- 2-27	66	98·0°F.	12	70·9	4·16	3·31	0·788	1411	1423	1383		
3- 3-27	—	—	12	79·1	3·88	3·26	0·832	1485	1481	1521		
8- 3-27	53	—	15½	86·4	3·48	2·83	0·805	1448	1451	1430		
	Average	..	..	75·7	3·84	3·24	0·836	1408	1397	1439		

Table 6. Women.

Subject.	Basal Metabolism. Calories per 24 hours. Gasometer Method.	Oxygen Method.	CO_2 Method.	% Variation of Average from Du Bois. Gasometer Method.	Oxygen Method.	CO_2 Method.
A	1252	1217	1398			
	1284	1297	1246	—8·4	—9·3	—4·6
	1268	1257	1323			
B	1408	1407	1434			
	1175	1189	1134			
	1313	1326	1281	—7·4	—6·9	—8·2
	1338	1341	1341			
	1309	1316	1298			
C	1374	1363	1426			
	1260	1204	1508	—18·5	—19·7	—12·8
	1202	1214	1167			
	1279	1260	1367			
D	1381	1345	1522			
	1478	1458	1561	+11·7	+9·3	+21·6
	1424	1387	1582			
	1428	1397	1554			
E	1095	1080	1161			
	1158	1152	1191	—12·6	—13·3	—8·6
	1126	1116	1176			
F	1120	1089	1248			
	1281	1264	1353	—8·0	—9·9	—0·5
	1200	1176	1299			
G	1135	1120	1203			
	1204	1201	1227	—12·2	—12·8	—8·8
	1169	1161	1215			
H	1198	1164	1333			
	1256	1224	1392	—14·3	—16·5	—4·8
	1227	1194	1362			
I	1419	1420	1537			
	1246	1221	1352	—9·9	—10·2	—5·3
	1249	1259	1223			
	1305	1300	1371			
J	1320	1345	1237			
	1181	1139	1357	—6·8	—8·5	+0·9
	1023	975	1223			
	1175	1154	1272			

TABLE 6. Women (continued).

	Basal Metabolism. Calories per 24 hours.			% Variation of Average from Du Bois.		
Subject.	Gasometer Method.	Oxygen Method.	CO_2 Method.	Gasometer Method.	Oxygen Method.	CO_2 Method.
K	1141	1138	1163			
	1116	1098	1194	—15·2	—16·0	—11·4
	1128	1118	1179			
L	1217	1163	1493			
	1222	1197	1330	—10·8	—13·7	+3·1
	1220	1180	1410			
M	1377	1400	1304			
	1294	1245	1223	—4·2	—5·2	—9·4
	1336	1322	1263			
N	1031	1031	1037			
	1407	1410	1412	—12·3	—12·5	—10·2
	1276	1260	1347			
	1238	1234	1266			
O	1409	1417	1388			
	1382	1385	1384	—10·6	—10·3	—11·3
	1396	1401	1386			
P	1409	1413	1407			
	1167	1157	1211	—17·1	—17·4	—15·4
	1069	1064	1097			
	1215	1211	1232			
Q	1333	1337	1328			
	1337	1357	1271	—7·2	—6·3	—9·6
	1335	1347	1299			
R	1344	1361	1294			
	1362	1368	1353	—13·4	—12·6	—15·4
	1353	1365	1323			
S	1236	1234	1254			
	1258	1201	1454	—7·0	—9·0	+0·7
	1272	1251	1373			
	1255	1229	1360			
T	1081	1070	1132			
	975	976	989	—33·8	—33·4	—35·9
	1112	1145	953			
	1056	1064	1024			
U	1145	1153	1123	—7·9	—7·2	—9·7

TABLE 6. Women (continued).

Subject.	Basal Metabolism. Calories per 24 hours.			% Variation of Average from Du Bois.		
	Gasometer Method.	Oxygen Method.	CO_2 Method.	Gasometer Method.	Oxygen Method.	CO_2 Method.
V	1487	1513	1401			
	1476	1499	1403			
	1291	1270	1378	—3·1	—2·5	—4·1
	1319	1322	1333			
	1393	1401	1379			
W	1066	1065	1082	—12·0	—11·8	—10·5
X	1046	1045	1059	—19·5	—19·5	—18·4
Y	1028	1128	1137			
	1070	1171	1174	—7·6	—7·5	—7·1
	1149	1150	1155			
Z	1407	1389	1503	—0·3	—1·5	+6·5
				—10·5	—10·9	—7·3

TABLE 7. Men.

Subject.	Basal Metabolism. Calories per 24 hours.			% Variation of Average from Du Bois.		
	Gasometer Method.	Oxygen Method.	CO_2 Method.	Gasometer Method.	Oxygen Method.	CO_2 Method.
1	1623	1624	1635			
	1677	1716	1428	+6·2	+7·2	—1·0
	1725	1753	1644			
	1675	1698	1569			
2	1155	1113	1323			
	1326	1270	1551			
	1091	1043	1320			
3	1508	1490	1590			
	1536	1533	1563	—13·3	—14·2	—9·3
	1486	1463	1584			
	1510	1495	1579			
4	1592	1528	1851			
	1630	1601	1752	—8·3	—9·8	—1·9
	1594	1609	1551			
	1605	1579	1718			
5	1624	1636	1593			
	1601	1609	1581	—10·5	—10·2	—10·6
	1507	1499	1548			
	1577	1581	1574			

TABLE 7. Men (continued).

Subject.	Basal Metabolism. Calories per 24 hours.			% Variation of Average from Du Bois.		
	Gasometer Method.	Oxygen Method.	CO_2 Method.	Gasometer Method.	Oxygen Method.	CO_2 Method.
6	1613	1604	1665	—14·4	—15·9	—12·7
8	1181	1179	1200			
	1183	1162	1275	—28·4	—29·2	—25·1
	1182	1170	1237			
9	1543	1522	1638	—3·3	—3·3	—1·9
	1522	1542	1461			
	1532	1532	1550			
10	1366	1330	1521			
	1486	1465	1578	—11·0	—13·4	—3·3
	1426	1387	1550			
11	1236	1208	1356	—18·0	—18·6	—14·0
	1286	1290	1281			
	1444	1435	1491			
	1322	1311	1376			
12	1364	1341	1464			
	1471	1457	1539	—12·1	—13·3	—6·8
	1418	1399	1502			
13	1347	1303	1527			
	1553	1520	1692	—11·1	—13·4	—1·4
	1425	1389	1575			
	1442	1404	1598			
14	1433	1436	1452	—12·6	—12·5	—11·5
15	1370	1346	1473			
	1416	1396	1500	—17·1	—18·4	—11·5
	1393	1370	1487			
16	1594	1569	1704			
	1496	1456	1665	—10·9	—12·3	—4·8
	1402	1395	1425			
	1497	1473	1598			
17	1515	1502	1574			
	1348	1304	1527	—14·8	—16·5	—7·7
	1432	1403	1550			
18	1464	1474	1443			
	1619	1615	1644	—8·8	—9·0	—7·0
	1540	1522	1626			
	1541	1537	1571			

TABLE 7. Men (continued).

	Basal Metabolism. Calories per 24 hours.			% Variation of Average from Du Bois.		
Subject.	Gasometer Method.	Oxygen Method.	CO$_2$ Method.	Gasometer Method.	Oxygen Method.	CO$_2$ Method.
19	1661	1637	1764			
	1704	1668	1851	—1·8	—3·1	+3·9
	1670	1663	1713			
	1678	1656	1776			
20	1734	1727	1776			
	1635	1626	1683			
	1606	1605	1626	—3·5	—3·2	—1·3
	1658	1653	1695			
21	1504	1488	1578			
	1541	1529	1635	—12·8	—13·6	—8·0
	1523	1508	1606			
22	1442	1408	1572			
	1631	1613	1711	—14·4	—16·1	—8·4
	1537	1515	1642			
23	1571	1580	1548			
	1442	1446	1455	—19·9	—20·3	—17·5
	1507	1472	1656			
	1507	1499	1553			
25	1460	1403	1689	—2·8	—5·7	+9·2
	1525	1492	1668			
	1493	1447	1678			
26	1652	1639	1713			
	1606	1599	1644	+4·2	+2·6	+6·4
	1640	1630	1695			
	1649	1623	1684			
28	1596	1594	1617			
	1682	1644	1800	—0·7	—1·2	+3·9
	1579	1561	1665			
	1619	1600	1694			
29	1401	1374	1518			
	1508	1533	1428	—12·9	—10·9	—9·9
	1375	1477	1491			
	1428	1461	1479			
30	1561	1513	1761	—5·5	—8·3	+6·7
31	1758	1744	1827			
	1763	1749	1854	+6·9	+6·2	+11·1
	1892	1884	1938			
	1804	1792	1873			

TABLE 7. Men (continued).

Subject.	Basal Metabolism. Calories per 24 hours. Gasometer Method.	Oxygen Method.	CO₂ Method.	% Variation of Average from Du Bois. Gasometer Method.	Oxygen Method.	CO₂ Method.
32	1622	1634	1590			
	1521	1518	1560	—9·1	—9·6	—6·1
	1485	1451	1629			
	1543	1534	1593			
33	1467	1457	1515			
	1362	1379	1311	—15·7	—16·1	—16·4
	1415	1418	1413			
34	1759	1762	1761			
	1609	1583	1722	—8·0	—8·6	—4·8
	1684	1672	1742			
36	2020	1990	1710			
	1901	1884	1575	+6·7	+5·4	—10·6
	2024	2002	1689			
	1982	1959	1658			
37	1695	1673	1794	—12·4	—13·5	—7·2
38	1385	1382	1398			
	1493	1507	1455	—17·3	—17·6	—15·4
	1213	1185	1329			
	1364	1358	1394			
39	1550	1573	1473			
	1584	1620	1458	—12·4	—11·2	—15·9
	1475	1482	1491			
	1536	1558	1474			
40	1447	1451	1446			
	1550	1550	1566	—14·6	—14·5	—14·1
	1498	1500	1506			
41	2065	2074	2055			
	2134	2156	2067	+15·0	+15·2	+15·4
	2115	2093	2220			
	2105	2108	2114			
42	1646	1636	1695			
	1545	1560	1503	+2·0	+2·1	+2·1
	1596	1598	1599			
43	1378	1338	1539			
	1322	1225	1425			
	1327	1284	1476	—14·5	—15·8	—8·1
	1251	1354	1236			
	1320	1300	1419			

TABLE 7. Men (continued).

Subject.	Basal Metabolism. Calories per 24 hours. Gasometer Method.	Oxygen Method.	CO_2 Method.	% Variation of Average from Du Bois. Gasometer Method.	Oxygen Method.	CO_2 Method.
44	1516	1538	1446			
	1508	1517	1485	—3·3	—2·4	—6·3
	1512	1527	1465			
45	1574	1567	1614			
	1600	1570	1728	+2·7	+1·5	+8·1
	1587	1569	1671			
46	1245	1228	1320	—29·4	—30·3	—25·1
47	1385	1353	1518			
	1524	1510	1593	+0·2	—0·7	+5·8
	1518	1515	1548			
	1476	1459	1553			
48	1703	1667	1854			
	1789	1800	1758	—0·1	—1·2	+2·9
	1746	1733	1806			
49	1226	1238	1197			
	1180	1191	1147			
	1188	1196	1174			
	1198	1208	1173	—22·4	—21·8	—24·1
50	1408	1397	1439	—17·7	—18·3	—15·8
				—8·9	—9·6	—6·0

TABLE 8. Subject X.

Time	Resp. Rate	Volume Expired 14.4 Minutes	Depth Respiration	R.Q.	Pulse Rate	CC per Minute		Metabolism Calories in 24 Hours			Type of Respiration
						CO₂	O₂	Gasometer	CO₂	O₂	
18-11-26:											
10.25–10.39·4	10	50·8	353	0·779	77	132	168	1158	1120	1167	Normal
11.5 –11.14·4	20	130·0	443	1·20	69	240	206	1494	2037	1432	Increased Rate and Depth
11.30–11.44·4	10	36·8	256	0·744	69	95	127	866	806	882	Normal
11.53–12.2	68	162·4	166	1·05	74	208	194	1409	1766	1348	Increased Rate
25-11-26:											
10.25–10.39·4	10	46·0	319	0·848	69	125	146	1020	1061	1015	Normal
10.55–11.9·4	10	99·5	691	1·19	70	224	187	1355	1900	1299	Increased Depth
11.40–11.54·4	10	45·6	317	0·788	65	122	154	1061	1036	1070	Normal
12.3 –12.17·4	30	90·0	210	0·954	65	160	166	1193	1358	1154	Increased Rate
30-11-26:											
10.32–10.46·4	9	44·7	345	0·841	67	126	148	1036	1067	1031	Normal
10.50–11.4·4	9	66·4	407	0·971	64	167	175	1261	1452	1214	Increased Inspiration
11.35–11.49·4	9	45·5	351	0·814	68	132	161	1118	1121	1120	Normal
11.51–12.5·4	10	94·5	656	1·09	72	212	192	1392	1840	1331	Increased Expiration
1-12-26:											
10.40–10.54·4	10	51·4	357	0·814	74	141	171	1189	1194	1191	Normal
11.5 –11.19·4	10	49·6	344	0·792	70	136	170	1174	1152	1182	Normal
11.30–11.44·4	10	81·3	565	0·949	70	187	196	1406	1591	36	Increased Inspiration

TABLE 9. Subject 50.

Time	Resp. Rate	Volume Expired 14.4 Minutes	Depth Respiration	R.Q.	Pulse Rate	CC per Minute CO$_2$	CC per Minute O$_2$	Metabolism Calories in 24 Hours Gasometer	Metabolism Calories in 24 Hours CO$_2$	Metabolism Calories in 24 Hours O$_2$	Type of Respiration
23-11-26:											
10.27–10.41·4	12	71·7	415	0·846	67	171	200	1399	1451	1390	Normal
11.39–11.53·4	11	95·0	600	0·851	60	189	212	1497	1605	1473	Increased Depth
12.6 –12.20·4	29	99·8	239	0·674	63	156	227	1534	1324	1577	Increased Rate
26-11-26:											
10.21–10.35·4	10	78·2	543	0·882	67	177	199	1403	1503	1383	Normal
10.45–10.55	10	145·8	1013	1·27	66	287	225	1634	2436	1564	Increased Depth
11.26–11.40·4	9	62·9	485	0·735	66	150	203	1376	1273	1411	Normal
11.49–12.2·4	28	110·9	275	0·800	62	192	238	1641	1630	1654	Increased Rate
2-12-26:											
10.15–10.29·4	10	76·9	534	0·889	58	178	199	1404	1511	1383	Normal
10.37–10·48	10	120·7	838	1·03	61	261	249	1810	2215	1730	Increased Inspiration
11.16–11.30·4	12	81·0	468	0·903	55	191	210	1488	1621	1459	Normal
11.38–11.50	12	127·0	735	1·11	61	273	244	1770	2318	1695	Increased Expiration

TABLE 10.

Effect of Depth of Respiration on B.M.R.

Rate of respiration.	% Increase in lung ventilation.	Alteration of R.Q.	% Increase in Basal Metabolic Rates.		
			Gasometer Method.	Oxygen Method.	Carbon dioxide Method.
12–11	32%	·85–0·85	7%	6%	11%
9– 9	48%	·84–0·97	22%	18%	36%
9– 9	52%	·82–0·99	14%	10%	31%
12–12	56%	·90–1·11	19%	16%	43%
10–10	57%	·89–1·03	28%	25%	46%
10–10	64%	·79–0·95	20%	15%	32%
10–10	86%	·88–1·27	16%	13%	62%
9–10	107%	·81–1·09	25%	19%	64%
15–15	112%	·81–1·23	21%	16%	77%
10–10	116%	·85–1·19	33%	28%	79%
9– 9	170%	·80–1·27	40%	33%	111%

Effect of Rate of Respiration on B.M.R.

Rates of respiration.	% Increase in lung ventilation.	Alteration of R.Q.	Depth of Respiration.	% Increase in B.M.R.		
				Gasometer Method.	Oxygen Method.	CO_2 Method.
12–29	39%	·85–0·67	415–239	10%	13%	—9%
9–28	76%	·74–0·80	485–275	19%	17%	28%
10–30	100%	·79–0·95	317–210	12%	8%	31%
12–25	118%	·83–1·22	458–479	8%	4%	53%
10–68	210%	·78–1·05	353–166	22%	15%	57%

TABLE 11. Subject X (7-3-27).

Time	Venous* Alveolar		R.Q.	Respiration	Volume Expired in		Depth Respiration CC	Metabolism			CC per 5 Minutes		Pulse Rate
	CO_2	O_2			5 Minutes	14.4 Minutes		Gasometer Method	O_2 Method	CO_2 Method	CO_2	O_2	
10.40 a.m.	6·28	12·06											68
10.44–10.58·4			0·801	9	16·5	47·6	368	1017	1021	1009	594	735	68.68.68.68
11.0	6·15	13·04											
11.8–11.13			1·30	9	39·2	—	784	1367	1307	2088	1230	941	70
11.13½	4·43	16·46											
11.14–11.19			1·30	9	43·1	—	958	1341	1282	2064	1216	923	
11.19½	4·10	15·89											
11.20–11.25			1·21	9	50·8	—	1129	1572	1503	2241	1320	1082	70
11.25½	3·71	15·53											
11.8–11.23			1·27	9	44·3	127·8	956	1427	1364	2131	1255	982	

*Last portion of expired air after holding the breath 30 seconds.

TABLE 12. Subject 50 (3-3-27).

Time	Alveolar		R.Q.	Respiration	Volume Expired in		Depth Respiration CC	Calories per 24 Hours. Metabolism			CC per 5 Minutes	
	CO_2	O_2			5 Minutes	14.4 Minutes		Gasometer Method	O_2 Method	CO_2 Method	CO_2	O_2
9.30 a.m.	5·49	14·41	0·800									
9.40–9.54·4			0·832	12	27·5	79·1	458	1485	1480	1520	896	1066
9.56	5·47	14·42	0·802									
10.10–10.15			1·22	25	55·9	—	457	1813	1732	2611	1538	1247
10.15–10.20			1·31	25	65·2	—	522	1532	1458	2390	1408	1054
10.20–10.25			1·13	25	58·3	—	466	1481	1417	1988	1171	1019
10.10–10.25			1·22	25	59·9	172·3	479	1609	1536	2330	1372	1107
10.25½	3·66	17·19	0·960									

TABLE 13. Subject X (9-3-27).

Time	Alveolar		R.Q.	Respiration	Volume Expired in		Depth Respiration CC	Metabolism			CC per 5 Minutes		Pulse Rate
	CO_2	O_2			5 Minutes	14.4 Minutes		Gasometer Method	O_2 Method	CO_2 Method	CO_2	O_2	
9·35 a.m.	5·56	13·95	0·749										
9.41–9.54·4			0·822	9	16·6	47·8	369	1075	1074	1088	641	773	71.72.69
	Venous*												
9.56	6·40	11·72											
10.2–10.7			1·07	9	25·5	—	567	1236	1182	1557	917	850	70
10.7–10.12			0·98	9	25·5	—	567	1123	1079	1292	761	777	66
10.12–10.17			0·907	9·5	24·7	—	521	1320	1288	1428	841	928	68
10.2–10.17			0·986	9	25·2	72·7	551	1226	1183	1426	840	852	
	Venous*												
10.17	5·40	13·73											
10.19–10.29			0·705	9	14·7	42·3	326	1047	1080	940	554	776	69

*Last portion of expired air after holding the breath 30 seconds.

TABLE 14. Subject 50 (8-3-27).

Time	Alveolar		R.Q.	Respiration	Volume Expired in		Depth Respiration CC	Calories per 24 hours. Metabolism			CC per 5 Minutes		Pulse Rate
	CO_2	O_2			5 Minutes	14.4 Minutes		Gasometer Method	O_2 Method	CO_2 Method	CO_2	O_2	
10 a.m.	4·66	15·53	0·823										
10–10.15			0·805	15·5	30·0	86·6	439	1448	1453	1429	842	1046	56 53
	Venous*												
10.15½	6·10	11·7											
10.23–10.28			1·27	15·5	64·2	—	828	1931	1846	2865	1688	1329	58
10.28–10.32			1·26	15·5	66·1	—	853	1701	1625	2503	1474	1170	
10.33–10.38			1·16	15·5	61·5	—	794	1646	1572	2236	1317	1312	
10.23–10.38			1·23	15·5	63·9	184·1	828	1759	1681	2535	1493	1210	58
	Venous*												
10.38½	3·68	15·77											

*Last portion of expired air after holding breath 30 seconds.

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A NEW METHOD FOR THE PREPARATION OF BACTERIOLOGICAL CULTURE MEDIA CONTAINING AGAR

by

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(Submitted for publication 4th August, 1927.)

Perhaps among all classes of culture media used by bacteriologists there is none more widely employed than the series having agar-agar as a base. Yet despite its universality the methods of preparation have left much to be desired, the chief problem being its clarification, which was only accomplished satisfactorily to the detriment of the nutritive value of the media and with considerable loss of bulk.

It has been experimentally established that the lower the temperature of filtration of an agar medium, below 100°C., the less nutritive the resultant medium; also the less heating to which the medium has been subjected the higher the growth factor of the end product.

The following method was devised to give a clear medium from unwashed agar filtered above 100°C. There is little heating, but the method is simple, and gives uniform results, with little loss of nutritive value and bulk.

The method involves the making of two stock preparations, one a clear agar jelly, and the other a nutrient broth.

The clear agar jelly is prepared as follows:

Two pieces of fine gauze are cut about 1 foot square. From a roll of cotton wool a piece about 8 or 9 inches square and a few millimetres thick is carefully removed, taking care that there are no thin patches, and is laid between the pieces of gauze. The centre is then pushed down into a litre Erlenmeyer flask, and the required amount of agar, in a granular condition, is introduced. The pad is then formed into a bag, with the agar in the centre, and tied firmly with string at the mouth. The excess gauze is then cut off, and the bag suspended in the flask by means of the strings, which are conveniently held by a rubber band around the neck of the flask (*vide* figure).

Seven hundred and fifty c.c. of distilled water are then poured into the flask, and the bag allowed to float loosely in the water. It is now autoclaved for 25 minutes at 120°C. Allow to cool to 100°C., and remove from autoclove. Raise the bag up towards the neck of the flask clear of the liquid, and replace in the autoclave for 10 minutes at 110°C. to allow all the agar to drain from the bag. When cool remove the empty bag from the flask, leaving the clear agar jelly.

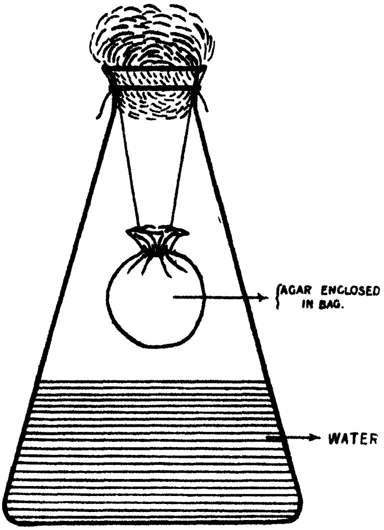

Figure 1.

The reaction may then be adjusted, and the nutrient broth, filtered and adjusted to the same reaction, added.

There is approximately 8% to 10% loss of agar, which may be allowed for, and a small loss of water, which can be made up where accurate measurements are required.

In actual practice, in making ordinary 2·5% nutrient agar the procedure is as follows:

For convenience in handling, the medium is made up in 1 litre lots. To make 1 litre of a 2·5% agar medium, 27 gm. of agar are used. This is dissolved in 750 c.c. of distilled water by the above method. The nutrient broth is prepared in quadruple strength by dissolving the meat extract (5 gm.), sodium-chloride (5 gm.), and peptone (10 gm.) in 250 c.c. of distilled water. heating for 20 minutes at 100°C., adjusting the reaction (to pH 7·3-7·4), and filtering through paper.

The 250 c.c. of quadruple strength broth are then added to the 750 c.c. of agar jelly, and the medium tubed and sterilized.

The agar jelly is conveniently prepared in batches of several flasks, each containing 750 c.c. These may be kept in stock, and when required for use 10 minutes at 120°C. is sufficient to liquefy the jelly.

OCCURRENCE OF *ONCHOCERCA GIBSONI* (WORM NODULE) IN CATTLE IN GIPPSLAND, VICTORIA

by

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(Submitted for publication 15th November, 1927.)

Up to the present time it has been thought that cattle bred in Victoria were entirely free from infestation with this parasite; indeed, the parasite has not to my knowledge been reported from places further south than round about Eden, on the south coast of New South Wales.

A few months ago the occurrence of the parasite in cattle bred and reared in South Gippsland was brought to my notice by Mr. D. Poole, the Agricultural Department's stock inspector for this area, and with his guidance I was enabled to see several dairy herds in which one or more members were found to be infected on manual examination. From one of these cows a nodule lying in the subcutaneous tissue of the chest wall just behind the right elbow, and about the size of a walnut, was excised and examined. On section it presented the characteristic appearance, and sufficient of the parasite was dissected out for purposes of identification as of the *Onchocerca* genus.

A second visit was undertaken recently (November 9) by Dr. Georgina Sweet, Mr. George McLennan, B.V.Sc., Mr. D. Poole, and myself. A number of farms in the neighbourhood of Foster were visited, and plentiful evidence of the occurrence of worm nodules was obtained. On one dairy farm, with a herd of thirty animals, more than half were palpably affected.

So far as Mr. Poole's survey has gone, nodules have been found in native-bred and reared cattle along a strip of coast country centering on Foster, about 60 miles long, and going back inland for about 12 miles.

Further work is now being undertaken in the district by Mr. G. McLennan, with a view to determining the limits of the distribution, the biting flies prevalent in the area, and, if possible, experimental methods of transmission of the parasite.

FURTHER OBSERVATIONS ON THE LIFE HISTORY OF THE EYE WORM OF POULTRY

by

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(Submitted for publication 1st September, 1927.)

Fielding (1926) has shown that the Surinam cockroach, *Pycnoscelus (Leucophaea) surinamensis* L., is responsible for the transmission of the eye worm *(Oxyspirura parvovum)* of poultry in Queensland. He stated that the cockroach picked up the larvae, but on further experimental work he has been unable to provide a continuity of larval life under cultural conditions. He has shown that when the infected cockroach is eaten up by the fowl, it does not pass beyond the crop before the contained worms liberate themselves, and pass up through the oesophagus into the mouth, and thence through the infra-ocular sinus or naso-lachrymal duct into the eyes, reaching their destination in a few minutes.

Influence of heat in the liberation of larvae.

Owing to the fact that the larvae take such a short time to reach the eyes, it was thought that the influencing factor in their liberation could not be due to any digestion of the roach, as this was practically nil. It was considered that possibly temperature was the main factor in their release, by simply providing a stimulus. To test this a few experiments were conducted by placing intact cockroaches in water heated to about 35°C. to 37°C., and it was seen that almost immediately the larvae began to pass through the thinner portions of the body, i.e., around the limbs, etc.

Influence of Humidity on Egg Laying.

From a series of daily examinations of the faeces and the mucous of the mouth it was noted that although a few eggs could be found on most days, there was a definite increase in their numbers on the approach of or during rain.

Hatching of the Eggs under External Conditions.

A series of experiments was conducted with the object of noting whether the eggs would hatch and the larvae live under controlled conditions. The media used were variable. It was found that in some cultures a fairly large percentage hatched, and that in some cases hatching was delayed up to a maximum time of 25 days from the start of the experiment. Soon after obtaining their freedom, however, the larvae became quiescent, and on keeping them under constant observation no movement was noted; it was consequently decided they were dead. This series was combined with observations on egg laying, longevity of the females, etc. The results are enumerated in Table 1.

TABLE 1.

Series.	Cultural Solution.	Number of adult females used.	Longevity of females.	Eggs passed on days.	Living larvae seen on days.	Remarks.
A	Water	14	Few hours	—	—	Adults burst. Dead larvae seen on 4th, 6th, 13th, and 17th day
B	0.9% Sod. Chloride	21	2–6 days	2nd–7th	5th–25th	In some experiments no larvae were seen
C	1.4% Sod. Chloride	4	3–8 days	2nd onward	8th–15th	In two experiments no larvae seen
D	0.9% Sod. Chloride + Eye Fluid	2	4 days	2nd–3rd	9th	
E	0.9% Sod. Chloride + Eye Fluid 1 drop to each c.c.	11	3–6 days	2nd onward	11th–17th	In two cultures the worms were teased. In three cultures no larvae were seen. In one culture no eggs were passed
F	1.0% Sod. Chloride 0.5% Sod. Phosphate 0.1% Sod. Bicarbonate in Aqua dist.	5	3–5 days	3rd onward	4th–11th	In two cultures no larvae were seen. In two cultures the worms were teased
G	1% Citrated fowl blood	3	3 days	2nd	5th	In one culture no eggs were passed
H	Earth moistened with water	5	Few hours to 5 days	4th	3rd	In four cultures the adults burst, liberated eggs showed larvae on 3rd day. Other adult passed eggs on 4th day but showed no development
I	Fowl faeces+Sand+Saline	12	Broken up	—	3rd–4th	The cultures were made of egg free faeces sterilized
J	Sterile fowl faeces, Saline and Charcoal	10	Broken up	—	5th & 7th	In three cultures no larvae were noted
K	Bread moistened with Saline	9	Broken up	—	3rd	Larvae in two experiments only, eggs seen in other

Free Larvae in the Oviduct of the Female.

The occurrence of larvae free in the oviduct was noted on a number of occasions while conducting the series tabulated below. It was interesting to note that although the females concerned were frequently laying eggs in the cultures, these larvae (in some cases) did not pass out, but remained alive in the oviduct for varying periods up to a maximum, in one female, of 5 days. In one case the larvae were alive on the death of the parent worm.

Experiments with Guinea-pigs.

In view of the finding that the development of larval nematodes on gaining access to an accidental host is generally arrested, and the third stage larvae remain in this stage, and proceed to re-encapsulate (Seurat), it was considered of interest to test this by introducing the eye worms to a guinea-pig, and at the same time to note whether they would find their way into the eye, and also, when deliberately introduced into the eye, whether development was arrested or not.

Experiment 1. A large number of eye worms (40 to 50) in the third stage were placed in the mouth of a guinea-pig, which was kept under observation for six weeks, but no evidence of worms in the eyes or re-encapsulation was found.

Experiment 2. Owing to the smallness of the eye cavity, it was decided to use only a limited number of the worms (about 12), which were placed in the left eye; thereafter the worms were extracted at varying intervals for the purpose of noting whether development kept pace with worms in their normal habitat. It was found that the parasites in this situation developed normally (so far as the experiment went), but apparently they could not find their way from one eye to the other, as none were seen except in the original location. The measurements, which compared favourably with those from the chicks and ducklings, are given in Table 2.

TABLE 2.

Day.	No. of Worms.	Measurements in mm.	Remarks.
6th	3	6·55, 6·65 & 7·37 x 0·19 to 0·24	Worms moulted.
8th	2	6·18 & 6·37 x 0·19	
12th	3	5·28, 6·8 & 7·0 x 0·18 to 0·22	
16th	2	9·24 & 8·84 x 0·24	Apparently moulting.
26th	1	8·22 x 0·24	Development advanced.

Preliminary to Experimental Infection of Cockroaches.

Since the majority of local poultry yards are infected with eye worms, and in consequence of a very large percentage of the cockroaches being infected, it became necessary to find an uninfected yard with plenty of cockroaches. This was difficult, since yards uninfected with eye worms were usually almost barren of cockroaches. About 800 roaches were examined from different parts of the town, but batch after batch had to be discarded owing to infection. After some weeks of patient work examining the body cavity for the presence of capsules and larvae, and the alimentary tract microscopically for the presence of eggs or first stage larvae; a batch was obtained which showed no infection in about 100 cockroaches examined, and it was considered that this lot could be used for the purpose of experimental infection.

Experimental Observations on Infection.

Experiment 3 was carried out with the object of ascertaining the time taken from the ingestion of the eggs of the worm to the time the larvae are infective. For this purpose a large batch of cockroaches were starved for three days. They were segregated in a large museum jar containing sterilized earth and a few dried leaves for hiding-places; bread soaked with physiological salt solution containing a large number of eggs, which had been taken from teased female worms, was then introduced into the jar. The bread was noted on the third day to contain a few living larvae, and was all eaten up by the fourth day. The results of examinations are tabulated below.

Experiment Started.

Day.		
1st.	Nymph and female examined.	Fully and under-developed eggs seen in gut.
	Bread examined.	Only eggs observed.
2nd.	Bread examined.	Only eggs observed.
3rd.	Nymphs and males examined.	Eggs and larvae measuring 235–260μ in length and 12–13μ in diameter seen in gut.
	Bread examined.	Eggs and larvae similar to above.
4th.	Bread consumed.	
6th.	Nymphs examined.	Eggs, uncapped eggs, and larvae seen in gut, one larva just emerging from egg-shell, which took 3 hours to get clear; this larvae measured 250 x 13μ. No larvae seen in body cavity.
10th.	Nymph and female examined.	The nymph showed larvae in gut measuring 235–250μ by 12–14μ. The female showed larvae free in body cavity, measuring 327–360μ by. 18–20μ.
13th.	Nymphs examined.	No larvae found in gut, but larvae measuring 320–360μ by 18–20μ were observed in body cavity.

Experiment Started. Day.		
17th.	Male, female, and nymphs examined.	Capsules measuring 273 by 364μ in diameter; the contained larvae, measuring from 485 to 600μ by 26 to 28μ, were quiescent.
18th.	Nymphs examined.	Capsules and worms similar to above. Larvae quiescent.
25th.	Nymphs examined.	Capsules found measuring 295 to 309μ by 360 to 436μ; the contained worms measured 0·63 mm. to 1·01 mm. long by 0·028 mm. to 0·035 mm. in diameter.
32nd.	Nymphs examined.	Capsules found containing larvae measuring 1·9 mm. to 2 mm. long by 0·07 mm. to 0·08 mm. in diameter.
39th.	Nymphs examined.	Capsules found containing worms measuring 3·4 mm. to 3·8 mm. long by 0·118 mm. to 0·127 mm. in diameter. (See Experiment 4.)
46th.	Nymphs examined.	Capsules found containing worms measuring 4·6 mm. to 4·9 mm. long by 0·127 mm. to 0·145 mm. in diameter. (See Experiment 5.)
52nd.	Nymphs examined.	Capsules found, worms measured 4 mm. to 5·3 mm. long by 0·127 mm. to 0·145 mm. in diameter. (See Experiments 6, 7, and 8.)

Attention is here drawn to the occurrence of two frequently, and sometimes three, larvae in the one capsule.

Experiment 4. Chick one week old given a large number of capsules from roaches which had been fed with bread containing eggs 39 days previously. No eye worms were discovered in the eyes of the chick. Result negative.

Experiment 5. Broken roaches (infected 46 days previously), containing large number of capsules, given to a two weeks' old chick. Result negative.

Experiment 6. Chick three weeks old given 2 nymphs (infected 52 days previously), containing a large number of capsules. Worms observed on the tongue, roof and floor of mouth 4 minutes afterwards, but were not seen in the eyes until 45 minutes had elapsed. Result positive.

Experiment 7. Similar to above, with 3 nymphs. Worms in the mouth 4 minutes after introduction and inside of the eyes after 6 minutes. Result positive.

Experiment 8. Chick three weeks old given four unbroken roaches after anaesthetizing (infected 52 days previously). Worms were not observed in the mouth, but were seen passing through the naso-lachrymal duct into the eyes 20 minutes after introduction. Result positive.

Control chick to above experiments remained negative throughout.

Observations on Infection under Natural Conditions.

A young infected rooster which had been used for a previous experiment was introduced into a pen in which no infected fowls had been kept and no infected cockroaches had been found after a long series of prior examinations. Roaches were then examined from this pen at frequent intervals, and after 61 days capsules and worms from these roaches were given to a chick (Experiment 9). Worms were found in the eyes 15 minutes afterwards. Result positive after 61 days.

Experiment 10 to note whether the Worms are Infective to Pigeons.

A full-grown pigeon was given two cockroaches, one of which was dead, but contained a large number of capsules and free worms; the other roach was killed and opened to ascertain the presence of capsules. Eight minutes after introduction the worms were in the eyes. Result positive.

TABLE 3.

Observations on the Worms in the Cockroaches.

Days after Feeding.	No. of Worms.	Location.	Measurements.		Remarks.
			Length.	Diameter.	
3rd-10th	Many	Free in gut	245–260μ	12–14μ	1st stage larvae.
10th-14th	Many	Free in body cavity	320–360μ	18–20μ	1st stage larvae.
17th-18th	Few	In capsules	485–600μ	26–28μ	1st stage larvae.
25th	Few	In capsules	0·63–1·01mm.	0·028–0·035mm.	Moulting
32nd	2	In capsules	1·91–2·09mm.	0·073–0·082mm.	2nd stage larvae.
39th	4	In capsules	(1) 3·5mm.	0·127mm.	2nd stage larvae.
			(2) 3·8mm.	0·118mm.	
			(3) 3·7mm.	0·118mm.	
			(4) 3·4mm.	0·118mm.	
46th	4	In capsules	(1) 4·9mm.	0·145mm.	Moulting
			(2) 4·9mm.	0·145mm.	
			(3) 4·8mm.	0·136mm.	
			(4) 4·6mm.	0·127mm.	

Days after Feeding.	No. of Worms.	Location.	Measurements.		Remarks.
			Length.	Diameter.	
52nd	4	In capsules	(1) 5·3mm.	0·09mm.	3rd stage larvae.
			(2) 4·0mm.	0·1mm.	
			(3) 5·1mm.	0·09mm.	
			(4) 5·09mm.	0·1mm.	

Observations on the Worms from Introduction to Fowl to Egg Laying of Female.

This series was intended to supply information regarding the measurements at the different stages in continuation of the previous series; time taken from introduction of larvae to definitive host, to maturity, and egg-laying of females. For this purpose the experimental chicks and ducklings were used as reservoirs, from which the worms were withdrawn at varying intervals. The results are tabulated in Table 4.

TABLE 4.

Days after Introduction.	Number of Worms.	Source.	Measurements.		Remarks.
			Length in mm.	Diameter in m.m.	
1	1	Duckling	5·3	0·182	Moulting.
1½	1	Duckling	5·1	0·16	Moulting.
2	1	Duckling	5·5	0·136	Moulting.
3	1	Duckling	5·64	0·109	Moulted = 4th stage larva.
4½	1	Duckling	5·9	0·14	Buccal cavity increasing in size.
6	3	Duckling	5·22	0·14	Muscular oesophagus distinct.
			4·55	0·127	
			5·1	0·14	
7	1	Duckling	5·46	0·145	
8	3	Duckling	5·27	0·127	
			5·49	0·145	
			5·46	0·127	
9	2	Duckling	5·5	0·127	
			5·3	0·118	
12	5	Ducklings (2) Chicks (3)	5·5	0·145	Length of oesophagus, 0·819 mm.
			6·18	0·182	
			7·09	0·182	Distance to nerve ring, 0·182 mm.
			8·3	0·23	
			8·46	0·218	

Days after Introduction.	Number of Worms.	Source.	Measurements. Length in mm.	Measurements. Diameter in mm.	Remarks.
15	4	Duckling (1)	8·5	0·25	
		Chicks (3)	9·0	0·25	
			9·19	0·24	Moulting.
			7·18	0·24	
19	1	Chick	7·8	0·27	
26	2	Chick	5·8	0·1	
			9·0	0·24	
28	1	Chick	8·2	0·218	
38	2	Chick	4·9	0·127	
			7·6	0·25	Egg laying noted.

Attention is directed to the size of No. 1 worm in each case on the 26th and 38th days. These worms were obviously under-developed.

Distribution of Oxyspirura parvovum, O. mansoni, and Pycnocelus (Leucophaea) surinamensis.

That there is an intimate association between the known distribution of the eye worm, *Oxyspirura parvovum*, and the cockroach, *Pycnocelus (Leucophaea) surinamensis* in Australia has recently been pointed out by Fielding. Both vector and parasite are known to occur at Townsville, Cairns, Ayr, and Hughenden. Messrs. Conigrave and Pugh informed the writer that the worm occurs at Darwin, the cockroach having been recorded previously from that locality.

It would appear also that the association between *O. mansoni* and the Surinam cockroach holds good, since both occur in China, Java, Brazil, and Honolulu. In Rabaul the eye worm occurs; and in New Ireland, which is closely adjacent, the cockroach has been recorded. This association is further accentuated by the fact that workers in Florida have recently found a cockroach implicated in the transmission of *O. mansoni*, which is regarded as the same species as the Australian vector.

SUMMARY OF CONCLUSIONS.

Although living larvae have been detected in the oviduct of the female worms (in which position they can live for some days), and notwithstanding the fact that living larvae have been noted up to a maximum of 25 days in culture, it would appear that there is no continuity of larval life under experimental conditions. It is now considered that normally the eggs hatch in the gut of the cockroaches. One larva was observed emerging from the egg two days after the last possible ingestion of eggs by the insect; larvae were noted in the gut six days after the last possible ingestion of eggs, and were found in the body cavity ten to thirteen days after the initial feed, or six to nine days after the last ingestion of eggs. Seventeen days after the initial feed, encapsulated larvae were found, which were approximately twice the size of newly-hatched larvae. It was found that the larvae were not infective 46 days after experimental feeding of the insect, but are so 52 days after, and that the female begins laying eggs 38 days after entering the definitive host, thus making the cycle of the parasite complete in approximately 13 weeks, though under more favourable conditions this time may probably be somewhat reduced. It was found also that on introducing infected cockroaches to pigeons the latter became infected, and that worms introduced into the eye of a guinea-pig were capable of continuing their development, but when introduced into the mouth of this animal they were incapable of reaching the eye.

Lastly, from the known distribution of the eye worms, *O. parvocum*, *O. mansoni*, and the cockroach *Pycnocelus (Leucophaea) surinamensis*, it would appear that there is a direct association between them.

ACKNOWLEDGMENTS.

In conclusion, I have to acknowledge my indebtedness to the Directors of the various Australian Museums, and Mr. Gurney (Government Entomologist of New South Wales), for supplying me with information regarding the distribution of the cockroach. I have also to thank Dr. A. H. Baldwin and Dr. G. M. Heydon for advice and assistance during the progress of this research.

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IN VITRO TESTS OF THE TOXICITY OF CERTAIN DRUGS FOR HYDATID SCOLICES

by

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(Submitted for publication 1st September, 1927.)

It has long been hoped that some method of treating hydatid disease of man, other than by surgical intervention, might be found, and from time to time claims have been made that certain drugs and also X-rays have been found effective as curative agents. Thus the use of Filix-mas, Kamala, tartar emetic, emetine, and arsenobenzol has been advocated. It cannot be stated that the evidence adduced in support of these claims has been such that conclusions of any value could be drawn from them. Moreover, Dévé (4, 5) has shown that in rabbits experimentally infested with hydatid cysts by the subcutaneous injection of hydatid "sand," the administration of large doses of Filix-mas and Kamala did not destroy the parasite or markedly inhibit its growth. In the same way Dévé and Payenneville (7, 8) found neosalvarsan and novarsenobenzol to be equally ineffective, while Dévé (3) and Dévé and Billard (6) found that hydatid scolices exposed to the action of X-rays, either before or after injection into the rabbit, were still capable of growth and cyst formation. Later, however, Dévé (5) obtained some indication that the vitality and regenerative powers of scolices exposed to X-rays of 20,000 R were markedly affected, so that he considers that further work along these lines is indicated.

The only controlled test of value in man was that of Fairley (9), who in two cases of hydatid disease injected amounts of tartar emetic approximating those administered in schistosome infestation. The drug apparently had no effect, as shown by subsequent operation in one case and complement fixation tests in the other, while in the former no trace of the drug could be recovered in the hydatid fluid. In spite of these observations the subject was considered to be worthy of further experimental investigation. It was decided to test the action of certain drugs on hydatid cysts *in vivo*, and as a preliminary *in vitro* tests on hydatid scolices were conducted, the results of which are embodied in this paper.

It was decided only to test drugs which are capable of being injected *in vivo* in relatively large doses, and which from their action on other parasitic infestations offer some likelihood of proving efficacious. The drugs selected were trypan blue, the value of which as a curative agent in various protozoan affections is established; acriflavine as representative of a different group of dyes, and because of its highly potent bactericidal action, at least *in vitro;* tartar emetic, whose toxicity for certain metazoan parasites is well proven, and of which another example has recently been provided by Itaki and Makino (11), who found it to have a marked effect upon the larvae of the *Dirofilaria immitis*, the heart-worm of the dog; and emetine, which again is largely used as a parasiticide, and which, moreover, was considered by Cawston (1), when used in conjunction with tartar emetic to have inhibited the growth of an hydatid cyst in man.

METHOD OF EXPERIMENTATION.

Hydatid scolices were obtained from hepatic and pulmonary cysts in the sheep, being drawn off under aseptic precautions. The cysts were first freed from the surrounding tissues, washed in alcohol, and the wall was then seared and pierced by a red-hot needle. After allowing a small quantity of the fluid to escape, and so wash out any contaminants, the contents were run into sterile containers. Approximately equal quantities of brood capsules and scolices were then added to varying dilutions of each drug, and incubated at 35°C. The drugs in all cases were dissolved in normal saline. Controls of scolices in normal saline and hydatid fluid were also cultured at 35°C., and were kept under observation for 48 hours, during which time a large majority remained alive and active.

The determination of the lethal time was made by pipetting off small quantities of scolices at stated intervals, and observing these in cavity slides on a warmed stage at 39°C. Those which had died were readily demonstrable by the loss of clarity of the various structures, such as the definition of the cuticle, and the dulling of the calcareous corpuscles and hooks, and more especially by the cessation of the active contractile movements seen in normal scolices.

ACTION OF DRUGS TESTED.

1. *Acriflavine.* The dilutions of the drug used were 1–200, 1–1,000, 1–2,000, 1–5,000, 1–10,000, 1–100,000.

After one hour's exposure to the action of the drug large numbers of scolices in 1–200 solution were dead, all others being active. After two hours all were dead in 1–200 solution, while in the other solutions the majority were alive. After three hours all were dead in 1–1,000 and many in 1–2,000 solutions. In four hours all scolices in 1–2,000 were dead, in six hours all in 1–5,000, and in eight hours all in 1–10,000. After eighteen hours the majority of the scolices in 1–100,000 were dead, but an occasional scolex was still moving feebly.

2. *Trypan Blue.* Dilutions of the drug used were 1–200, 1–1,000, 1–2,000, 1–5,000.

After one hour's exposure all scolices were alive, but few had evaginated. After two hours all scolices were alive, and about 25% in 1–200 solution had evaginated. In four hours all were alive, and the majority in 1–200 solution were evaginated, and a considerable proportion in 1–1,000. After eight hours all scolices were still contracting actively, and a large proportion in all solutions had evaginated. In sixteen hours almost all scolices in 1–200 solution were dead, but an occasional scolex was still moving, many were dead in 1–1,000 solution, but the majority were alive in 1–2,000 and 1–5,000 solutions. After twenty-four hours an occasional scolex still showed movement in 1–1,000 solution, while about 20% were alive in 1–2,000 and 1–5,000 solutions.

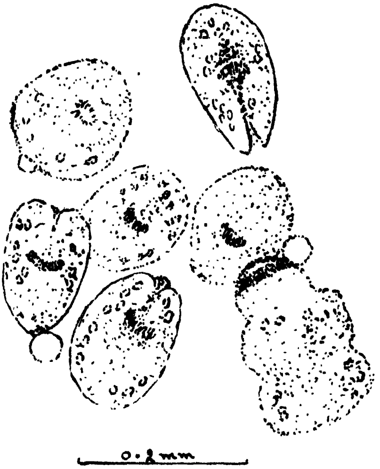

Figure 1. Scolices in Acriflavine, 1–5,000, after five hours' exposure, showing "spherules belleuses."

3. *Tartar Emetic.* The dilutions of the drug used were 1–100, 1–1,000, 1–2,000.

After two hours all scolices were alive and moving actively, and few were evaginated. In three hours many scolices in 1–100 solution were dead, those in the other solutions being alive. After four hours all in 1–100 were dead, while those in 1–1,000 were commencing to die, few of these scolices having evaginated. In six hours all scolices in the 1–1,000 solution were dead, as were a few in 1–2,000. In eight hours the majority in 1–2,000 were dead, while in ten hours all were dead. Only a small proportion of scolices in the various dilutions of the drug evaginated.

4. *Emetine.* Dilutions of the drug used were 1–100, 1–1,000.

After four hours all scolices were alive. After six hours a few scolices in 1–100 solution were dead, and in eight hours all scolices in this solution were dead. After fourteen hours the majority of the scolices in 1–1,000 solution were alive. Few scolices in either solution were evaginated.

Consideration of the above results reveals a striking variation in the action of the drugs on hydatid scolices. Acriflavine proved most toxic, a 1–1,000 solution being fatal in three hours, a 1–10,000 solution in eight hours, while even 1–100,000 killed the great majority of scolices in seventeen hours. When compared with that of acriflavine the action of trypan blue is surprising, scolices remaining alive and active in a 1–200 solution for sixteen hours, while after twenty-four hours an occasional scolex in 1–1,000 solution was alive, and a considerable proportion in 1–2,000. After acriflavine, tartar emetic was most rapidly fatal, a 1–1,000 solution killing all scolices in six hours. Emetine was relatively ineffective, a large proportion of scolices being alive and actively contracting after fourteen hours in a 1–1,000 solution.

As was to be expected, none of the drugs gave results comparable with those given by corrosive sublimate or formalin, which in a dilution of 1–1,000 and 1–200 respectively were found by Coutelen (2) to be fatal in two to three minutes.

It is not considered that the results obtained necessarily indicate the relative potency of the drugs *in vivo*, since, though acriflavine has a relatively high bactericidal potency *in vitro*, and might be considered to be equally valuable *in vivo*, doubt has been cast on its ability to inhibit bacterial growth *in vivo* by Meleny and Zau (12). On the other hand, Fairley (10) has shown that the toxicity of tartar emetic for schistosome cercariae is immeasurably increased in serum, and therefore probably *in vivo*, when compared with its action in aqueous solution *in vitro*. Moreover, it cannot be determined how far length of exposure to very dilute solutions of the drugs *in vivo*, as in a long course of administration in hydatid disease might not produce results at variance from those obtained after a relatively short exposure to high concentrations, as in the above experiments.

Other Phenomena Observed. As was also found by Coutelen (2) bacterial contamination is markedly deleterious to the existence of hydatid scolices *in vitro*, since when this is heavy death may take place in less than 24 hours, and no scolices are seen to survive for longer than 48 hours. Evagination of scolices appeared to be influenced to a considerable extent by the action of the drugs. Thus in the case of acriflavine it appeared that death in many cases took place so rapidly that only a small minority evaginated, as shown by the figure, while in trypan blue the process seemed to be hastened, the great majority evaginating in its dilutions before those in the control cultures. In emetine, on the other hand, a large majority of the scolices remained invaginated, though alive, after fourteen hours. Another indication of the toxic action of the drug, and the approaching death of the scolices was the detachment of the scolices from the everted wall of the brood capsules. In a few hours, in the higher strength acriflavine solutions, practically all were detached, while in trypan blue the great majority remained firmly attached by their pedicels to the wall.

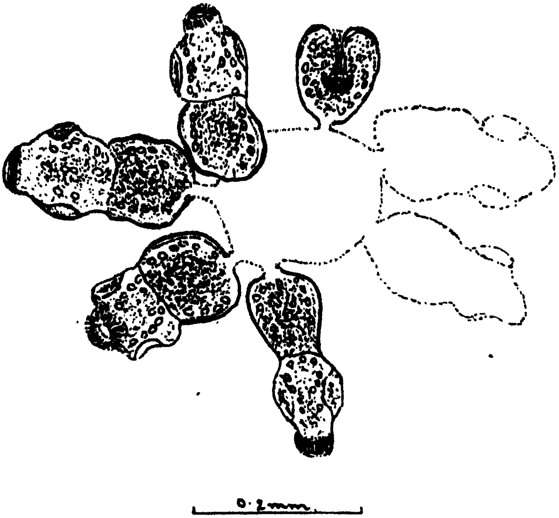

Figure 2. Scolices in trypan blue, 1–1,000, after six hours' exposure.

It was also observed that many scolices appeared to emit bubble-like objects from either the anterior pole or that at which the pedicel is attached. These without doubt are the "spherules bulleuses" described by Coutelen (2), of which the exact nature is not clear. Coutelen considers they may be either thrown out as a result of the change from the high intracystic pressure to that of the external *milieu*, which is probably less than the internal pressure of these scolices, or be an indication of the commencing degeneration of the scolex. From my observations the latter would appear to be the case, since their formation was seen to occur most numerously shortly before death. In acriflavine solution, 1–2,000, they would be observed in from two to three hours, while in trypan blue solutions they would not appear till very much later, and then only shortly before death. The bubbles were clear and structureless, and it was not possible to see the fine granulations which Coutelen has at times found.

SUMMARY.

The action of acriflavine, trypan blue, tartar emetic, and emetine on hydatid scolices was tested *in vitro*. Acriflavine proved most highly toxic, and then tartar emetic, emetine, and trypan blue in the order named.

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